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A STATISTICAL STUDY OF ABSOLUTE EOSINOPHIL CELL COUNTS IN HEALTHY YOUNG ADULTS USING LOGARITHMIC ANALYSIS

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BECAUSE of the present interest in circulating eosinophils in relation to adrenal function, we thought it worth while to find accurate statistical constants for eosinophils in healthy young men. In searching through the literature we were surprised to find considerable variation in reports of "normal" levels. These are shown in Table I.[1-9]

Two detailed studies were reported by Rud[9] and by Fisher and Fisher.[5] Rud studied 71 young healthy individuals (42 women and 29 men) throughout the year by repeated counts at random times. He found a range of eosinophils of from 40 to 230 cells per cubic millimeter. He calculated the mean level to be 126 cells. Eosinophil cell counts were made from capillary blood (taken from the ear lobe) and a 1:20 dilution in a single leukocyte pipette with Magdala red stain, using a Fuchs-Rosenthal counting chamber.

Fisher and Fisher[5] reported a more extreme eosinophil cell range in "normals" extending from 20 to 880 cells per cubic millimeter. The mean eosinophil level was 221 cells per cubic millimeter; this was almost one hundred cells greater than Rud's mean of 126 cells per cubic millimeter. Fisher and Fisher used Henkleman's[10] technique in making single eosinophil counts on capillary blood. They did not analyze their data statistically, and used hospital patients for obtaining "normal" values. Because the technique used by various investigators (as shown in Table I) are not above reproach, and because data on absolute eosinophil counts have been omitted from the recently published Handbook of Biological Data,[11] this study was undertaken to determine the range of eosinophils in young, healthy, adult males.

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TABLE I. NORMAL RANGE OF EOSINOPHIL CELLS

SOURCE	SUBJECTS	RANGE CELLS PER CUBIC MILLIMETER	MEAN	TECHNIQUE
1. Osgood[1]	597 individuals (ages up to 30 yr.)	0-400 0-980 (extreme counts)	140	Differential smear counts
2. Todd and Sanford[2]		50-400		
3. Kracke and Parker[3]		75-300		
4. Simpson[4]	31 individuals (25 men and 6 women)	50-400	150	Differential smear counts
5. Fisher and Fisher[5]	102 student nurses and 47 male patients	25-300 (most counts) 20-880 (range fasting pts.)	221	Hinkleman's technique, capillary blood
6. Roche and associates[6]	29 preoperative patients	11-440 (range of all counts)	155	Dunger's technique, venous blood, 1/20 dilution
7. Ham[7]		150-300 (most counts) 45-500 (extreme counts)		
8. Thorn[8]		100-250		
9. Rud[9]	71 healthy subjects 29 males, 42 females	40-230 38-221 41-236	126 121 129	Magdala red stain, capillary blood 1/20 dilution, Fuchs-Rosenthal chamber
10. Rud*	51 healthy subjects	54-465 (two standard deviations)	159	8 A.M. blood specimens
11. Present Study	42 healthy young adults	72-440 (two standard deviations)	179	Henneman variation of Randolph technique, venous blood, 1/10 dilution, series of six counts. Data treated statistically using Litchfield's log-probit technique

*Author's logarithmic treatment of Rud's data using Litchfield's technique.

METHODS

Selection of Subjects.—Forty-two healthy subjects were selected from a group of volunteer male medical students ranging in age between 21 and 34 years. The subjects gave no history of previous blood dyscrasia or allergic disease. They felt perfectly well when studied, and physical examination revealed no clinical evidence of disease.

Plan of the Study.—The data were collected throughout the year. Subjects carried out normal activities and consumed their normal daily diet.

Venous blood was taken at approximately 8:00 A.M., before breakfast, while they were fasting, and within one hour of awakening, every Monday, Wednesday, and Friday for two weeks. Without delay 5 ml. of blood were drawn by free venipuncture without tourniquet; the blood was collected into a dry glass vial containing heparin, mixed gently about fifty times, and immediately thereafter mixed at 60 r.p.m. for at least two minutes on a rotary pipette spinner to insure thorough mixing of the blood and heparin.

Technique of Eosinophil Cell Counts.—The Henneman[12] modification of the Randolph method[13] was used in counting eosinophils. Two calibrated leukocyte pipettes and four double Levy counting chambers (eight counting cells) were used to obtain each eosinophil count.

Statistical Treatment of Data.—Mean values and standard error were calculated using Fisher's[14] method.

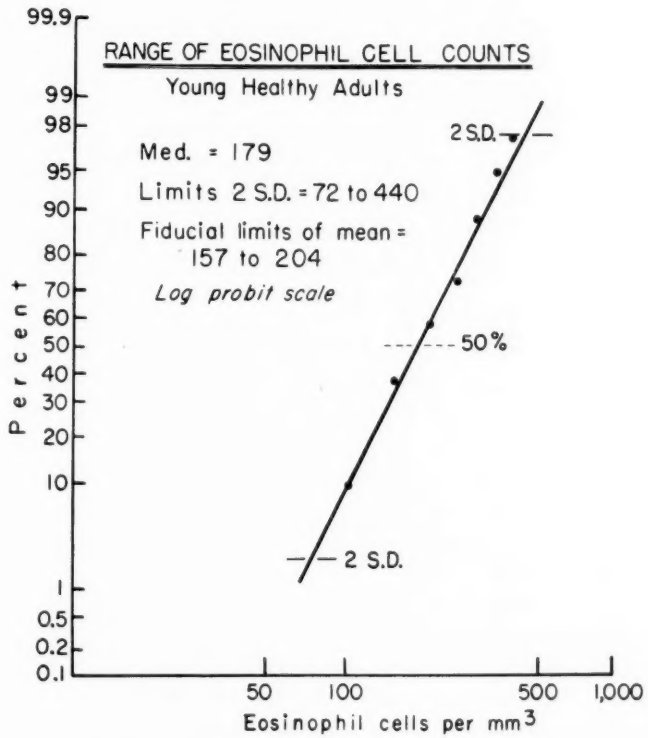

Fig. 1.—Range of eosinophil cell counts in 42 young healthy males plotted on log-probit graph paper. The points 50 per cent, 2 S.D., and −2 S.D. were obtained by analysis of data using Litchfield's[15] method (see text). The ordinate shows the cumulated per cent distribution of the sample population. The abscissa shows the eosinophil cell counts per cubic millimeter.

Table IV: Range of 50 Cell Increments of Eosinophils, the Number of Observations That Fall Within These Increments, and the Cumulative Observations of the Per Cent of These Observations on 42 Young Healthy Males.

RANGE	NUMBER OF OBSERVATIONS	CUMULATIVE OBSERVATIONS PER CENT OF TOTAL
0–50	0	0
51–100	4	9.42
101–150	12	37.99
151–200	9	59.43
201–250	6	73.71
251–300	6	88.00
301–350	3	95.14
351–400	1	97.52
401–450	0	
451–500	1	100.00

Details of Calculations as Stated in Text (Methods) Using Data from Table IV.—

I. $EC_{84} = 282$ = one plus probit

II. $EC_{50} = 179$ = logarithmic mean

III. $EC_{16} = 115$ = one minus probit

IV. "S" $= \dfrac{\dfrac{(I)}{(II)} + \dfrac{(II)}{(III)}}{2} = 1.57$

V. Number of individuals studied = N = 42

VI. F_{EC50} is obtained from nomograph # 1 of Litchfield[15] by extrapolation using (IV) and (V) = 1.14.

VII. The fiducial limits of the logarithmic mean (Median) $= \dfrac{(II)}{(VI)}$ and (II) × (VI) = 157 and 204.

TABLE II. ABSOLUTE EOSINOPHIL CELL COUNTS (PER CUBIC MILLIMETER VENOUS BLOOD) IN 42 HEALTY MALES

SUBJECT	FASTING (8 A.M.) EOSINOPHIL CELL COUNT PER CU. MM. TAKEN ON:—						MEAN EOSINOPHIL CELL COUNT PER CU. MM.
	MON.	WED.	FRI.	MON.	WED.	FRI.	
G. W.	292	336	341	275	220	252	286.00
J. P.	215	192	198	116	132	209	177.00
L. C.	253	193	110	143	176	110	164.16
V. D.	256	297	303	281	363	275	295.83
L. T.	203	154	176	215	182	143	178.83
R. C.	107	121	116	66	165	94	111.50
M. J.	182	187	198	187	189	198	188.50
R. M.	303	203	243	292	396	253	281.67
J. C.	176	149	45	182	116	143	135.16
G. A.	225	182	182	148	209	193	189.83
F. J.	209	279	253	221	203	127	216.33
S. S.	137	369	225	254	242	319	257.66
B. V.	176	176	204	215	247	198	202.66
G. J.	72	77	61	88	66	99	77.17
J. L.	363	308	407	264	302	317	326.83
H. M.	292	187	198	220	165	132	199.00
T. S.	82	93	165	107	165	164	142.66
H. U.	104	115	132	165	105	105	121.00
R. K.	178	284	237	198	208	156	210.16
G. J.	72	77	91	62	88	95	80.83
C. N.	324	289	154	261	189	231	241.33
G. M.	172	112	95	112	95	154	123.33
S. K.	444	489	469	461	401	466	455.00
R. K.	184	289	172	189	209	233	216.66
W. K.	56	72	72	56	47	45	58.00
H. S.	117	67	150	89	100	89	102.00
W. V.	278	311	317	278	275	334	298.83
G. S.	172	89	112	161	78	128	123.33
J. A.	123	123	139	117	110	200	135.33
J. K.	188	151	129	134	128	217	157.83
T. K.	348	400	317	333	345	383	354.33
J. T.	151	161	89	117	78	139	122.50
L. U.	256	95	261	195	283	178	211.33
B. J.	284	224	200	295	289	306	266.33
H. N.	200	95	129	206	161	184	162.50
J. L.	123	78	106	49	100	172	104.66
E. F.	240	223	161	117	123	150	169.00
F. F.	129	134	145	156	100	139	133.83
K. J.	89	117	89	95	98	95	97.00
B. L.	322	278	320	303	289	405	327.83
J. I.	150	167	128	100	109	100	125.66
B. U.	211	300	250	291	406	410	311.33

Litchfield's[15, 16] technique using logarithmic-probit graph paper (Fig. 1) was employed to calculate the logarithmic mean (median or EC_{50}), fiducial limits of the logarithmic mean, and the range of two standard deviations (plus or minus two probits) for chamber eosinophil cell counts.

Litchfield's method of analysis plots cumulated percentages of the sample population against their total eosinophil cell counts on log-probit paper. The cumulated percentages of subjects were plotted against fifty cell increments in the eosinophil cell count from 0 to 500 eosinophils. A straight line was drawn on the graph paper which fitted most closely to the individual plotted points.

EC_{50} is a point on the line which represents the eosinophil cell level below and above which 50 per cent of the subjects were found, and is the logarithmic mean or median. This is the intersection of the line and zero probits. EC_{16} represents a point on the line equivalent to minus one probit or one standard deviation. The eosinophil cell level of 16 per cent of the subjects will fall below this point.

EC_{84} is a point on the line which is equivalent to plus one probit. It represents the eosinophil cell level below which 84 per cent of the subjects will fall, and is equal to plus one probit or plus one standard deviation.

The range of minus and plus two standard deviations was obtained by reading the eosinophil cell count at the intersection of the line of plotted values and the line of minus and plus two probits.

The fiducial limits of the logarithmic mean (median or EC_{50}) describe the range within which there is a 95 per cent probability that the logarithmic mean eosinophil cell counts of an infinitely larger sample would fall. These values are obtained through the use of the formulas and nomogram of Litchfield.[15]

RESULTS

Two hundred fifty-two observations were made of the circulating eosinophils in the venous blood of forty-two young healthy adults (see Table II). The individual eosinophil cell counts ranged from 45 cells to 483 cells per cubic millimeter.

The cumulative data of all counts are tabulated in the appendix. Using Litchfield's method, the logarithmic mean (EC_{50}) was found to be 179 cells per cubic millimeter. Minus and plus one standard deviation by logarithmic analysis (EC_{16} and EC_{84}) were 115 cells and 282 cells per cubic millimeter, respectively. The range of two such standard deviations was from 72 cells to 440 cells per cubic millimeter. The fiducial limits of the logarithmic mean were calculated to be 159 cells and 204 cells per cubic millimeter, and Litchfield's[15] nomograph was used in the calculations.

The arithmetic mean eosinophil cell count was calculated to be 209 cells per cubic millimeter; the standard error of the mean was 15.9.

DISCUSSION AND CONCLUSIONS

In any study of eosinophil cell levels the accuracy and extent of sampling and counting are crucial factors as far as validity of data are concerned. The intrinsic error of chamber eosinophil counts using one pipette with a 1:10 dilution and four Levy chambers is pointed out in Table III.[18] In this table

TABLE III. INTRINSIC ERRORS OF CHAMBER EOSINOPHIL COUNTS USING ONE PIPETTE WITH 1:10 DILUTION AND FOUR 0.9 CUBIC MILLIMETER COUNTING CHAMBERS

TRUE EOSINOPHIL LEVEL OF SAMPLE	RANGE FOR 95 PER CENT OF CHAMBER COUNTS ON SAMPLE	COEFFICIENT OF VARIATION
25 cells/cu. mm.	8-42	34%
50 cells/cu. mm.	26-74	24%
100 cells/cu. mm.	65-135	18%
200 cells/cu. mm.	148-252	13%
400 cells/cu. mm.	323-477	10%

eosinophil cell levels of the venous sample are compared to the range of 95 per cent of chamber counts on that sample. Moreover, this intrinsic error in counting is increased when single chamber counts are made. In this study the validity of data was increased by using two pipettes and eight Levy chambers.[18, 19] For example, if only one leukocyte pipette is used to sample a blood specimen

containing 100 eosinophils, and if two Levy chambers are filled and counted, it is possible to obtain counts which range from 55 cells to 145 cells per cubic millimeter. With two pipettes and eight Levy chambers the counts would range from 77 cells to 123 cells per cubic millimeter.

The usual bell-shaped distribution curve was not obtained when the eosinophil cell counts were plotted arithmetically. The curve was skewed, and the peak was found to be to the left of the mean (see Fig. 2). This occurred because the distance between the mean and zero was limited. Naturally, plus or minus two standard deviations could not be calculated from the arithmetic plot.

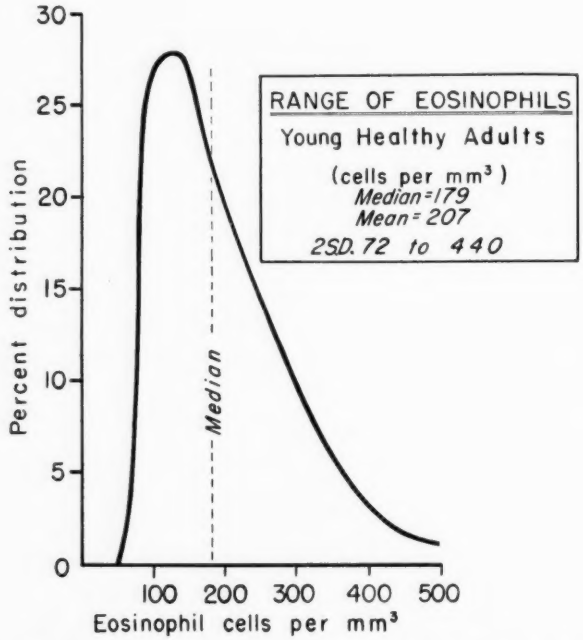

Fig. 2.—Range of eosinophil cell counts in 42 young healthy males plotted arithmetically. The ordinate shows the per cent distribution of the population. The abscissa shows their eosinophil cell counts per cubic millimeter.

Some biological data (e.g., blood bilirubin values)[20] do not follow a normal arithmetic distribution. However, when the logarithms of these data are plotted, a normal distribution may be found. To extract significant statistical data from eosinophil counts it was necessary to use logarithmic analysis, and in this study a graphic probit analysis as simplified by Litchfield was used.

A statistical re-evaluation and analysis were also made of all data on eosinophil counts accumulated by Rud[9] using Litchfield's method. In his group of 71 healthy young adults (42 women and 29 men) the logarithmic mean of eosinophil counts collected at different times of the day and night was calculated to be 119 cells per cubic millimeter, and the range of two standard deviations extended from 46 cells to 304 cells per cubic millimeter (see Table I). The above logarithmic calculations differ from Rud's arithmetic mean, of 126 cells

per cubic millimeter, and range of eosinophil cells, from 40 to 230 cells per cubic millimeter. The difference stems from the fact that Rud treated his data in an arithmetical manner. This accounts for the higher mean value he obtained, and also explains the narrow range of "normal" eosinophil counts he reported.

In our logarithmic treatment of Rud's[9] data, *of single eosinophil counts made at 8:00 a.m.*, from a group of 51 healthy individuals (35 women and 16 men) we calculated the range of two standard deviations to extend from 54 cells to 465 cells per cubic millimeter. A logarithmic mean of 159 cells per cubic millimeter was found. This finding parallels the results reported in this study.

With regard to data on eosinophil levels published elsewhere, two points need to be mentioned. First, it seems inadvisable to use patients to provide data for "normal" or "healthy" values, and second, it is well known that counts made from capillary blood are not as reliable as counts made from free-flowing venous blood. The data in this study (Table II) confirm previous reports,[9, 17] which show that there appears to be a tendency in the healthy individual to maintain a certain equilibrium in the eosinophil cell level despite variations in the leukocyte levels.

In conclusion, it is believed that this study presents an accurate range of eosinophils (two standard deviations) in healthy young adults which may be used as a "yardstick" to evaluate the existence of an eosinopenia or an eosinophilia in the peripheral blood.

SUMMARY

Eosinophil cell counts were made at 8:00 A.M. on free-flowing venous blood from forty-two fasting healthy young males during a two-week period. Six counts were made on each individual. The data from two hundred fifty-two counts were treated statistically using log-probit analysis and Litchfield's technique. From the statistically treated data the following calculations and observations were made: (a) The individual counts ranged from 45 to 483 cells per cubic millimeter. (b) The logarithmic mean eosinophil count was 179 cells per cubic millimeter. (c) The fiducial limits of the logarithmic mean (median) were 159 and 204 cells per cubic millimeter. (d) The range of two standard deviations extended from 72 to 440 cells per cubic millimeter. (e) The mean eosinophil count (arithmetical) was 207 cells per cubic millimeter. (f) The standard error of the arithmetical mean was 15.9.

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EPINEPHRINE EOSINOPENIA IN SURGICALLY INDUCED ADDISON'S DISEASE

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OBSERVATIONS made in our laboratory[1-3] and elsewhere[4, 5] have cast serious doubts on the clinical value of the four-hour eosinophil cell "response-test" to parenteral epinephrine. The test was developed by Recant and associates[6] and, at present, it is used by many physicians to estimate the functional activity and the integrity of the pituitary-adrenal system in man.[7-11]

The response to epinephrine is tested by counting circulating eosinophil cells before, and four hours after, injecting 0.3 mg. of epinephrine into the patient. According to Thorn and Forsham,[12] a fall of 50 per cent or more in circulating eosinophil cells indicates normal activity of the adrenal cortex or the anterior part of the pituitary gland. Unfortunately, most reports have been based on an incorrect interpretation of the test, which is quite different from the interpretation given by Thorn. In the literature, more often than not, a poor response to epinephrine, with little fall in the level of eosinophil cells four hours after injection, is accepted as evidence of disease or dysfunction of the hypothalamus, pituitary gland, or adrenal cortex.

Recant and her colleagues[6] suggested that during the test the epinephrine released eosinopenic adrenocortical hormone (s) into the blood stream through successive stimulation of the hypothalamus (by epinephrine), the anterior pituitary gland (by hypothalamic excitatory neurones), and the adrenal gland (by ACTH). However, in this communication observations are reported, on patients with bilateral complete adrenalectomy, which indicate that epinephrine eosinopenia can be induced in the absence of the adrenal gland.[13] This finding, coupled with data from statistical analyses of epinephrine response tests[1-3] lead us to conclude that the four-hour eosinophil cell response to parenteral injection of epinephrine should not be used in clinical practice to test the function or integrity of the pituitary system in man.

METHODS

Patient Material.—Five male patients were studied. These individuals were suffering with carcinoma of the prostate which had metastasized. Bilateral orchidectomy, followed by a two-stage, bilateral, complete adrenalectomy were done as therapeutic measures. The operations on the five patients were performed one, two, three, and five weeks before this

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study began. The patients were maintained in a eu-adrenal state by daily therapy with Cortone Acetate (cortisone acetate-Merck). Each day patient C. G. was given 6.25 mg. of cortisone at 6:00 A.M., 12:00 noon, and 6:00 P.M. Patient I. S. was given 12.5 mg. at 6:00 A.M. and 6:00 P.M.; and patient B. W. was given 25 mg. at 6:00 A.M. and 6:00 P.M. Patients B. O'H. and J. M. were given 25 mg. cortisone at 6:00 A.M., at 12:00 noon and at 6:00 P.M.

Technique of Testing.—Pre-test (zero hour) venous blood samples for control eosinophil cell counts were taken between 8:45 A.M. and 9:00 A.M., immediately after breakfast had been eaten. At 9:00 A.M. one of three substances was injected into the patients. This was a subcutaneous injection of saline (the placebo control), or a subcutaneous injection of 0.3 mg. of epinephrine, or an intramuscular injection of 40 mg. potent, pre-tested ACTH (Armour) (the ACTH control). As we have shown that inadequate eosinophil responses to test materials may occur if tests are run on successive days,[3] each test was separated from the next by at least four days.

After the injection the patients stayed quietly at their beds and four hours later a second sample of venous blood was taken (1:00 P.M.), lunch being eaten between 12:00 noon and 12:30 P.M. The patients did not know what they were getting, but reacted in the usual way to the injection of epinephrine.

Originally the four-hour eosinophil cell response tests to epinephrine or corticotropin were conducted in our laboratories while the patients were fasting, but since Best and Samter[19] demonstrated that fasting per se caused a marked drop in circulating eosinophils at midday, we have allowed our patients and healthy subjects to eat their breakfast and lunch during testing. This does not interfere with the eosinopenic effect of ACTH, adrenocortical hormones or epinephrine.[15]

Technique of Counting Eosinophil Cells.—This has been described in detail elsewhere.[3] In brief, the eosinophil cells are stained by the Henneman modification[16] of the Randolph method,[17] and for each sample of blood two leukocyte pipettes and eight Levy counting chambers were used to obtain an individual count. Statistical significance was insured by reference to tables and charts published elsewhere.[3, 19]

RESULTS AND DISCUSSION

In the five patients with surgical Addison's disease, reported here, there was excellent evidence that the pituitary-adrenal axis was completely interrupted. First, the glands were removed in toto at operation (as shown by gross and

TABLE I. THE EFFECT OF PARENTERAL INJECTION OF EPINEPHRINE (0.3 MG.), CORTICOTROPIN (ACTH) OR SALINE PLACEBO ON THE CIRCULATING EOSINOPHILS OF FIVE PATIENTS WITH CARCINOMA OF THE PROSTATE, TREATED BY BILATERAL, COMPLETE ABLATION OF THE ADRENAL GLANDS, AND MAINTAINED IN A EU-ADRENAL STATE WITH ORAL CORTISONE ACETATE

PATIENT AGE	ORAL CORTISONE ACETATE MG./DAY	CIRCULATING EOSINOPHIL CELL COUNTS (CELLS PER CUBIC MILLIMETER BLOOD) BEFORE AND 4 HOURS AFTER INJECTION OF:					
		EPINEPHRINE		ACTH		SALINE PLACEBO	
		0 HR.	4 HR.	0 HR.	4 HR.	0 HR.	4 HR.
C. G. 62 yr.	18.75	161	35	92	89	81	77
I. S. 60 yr.	25	41	19	28	39	31	25
B. W. 47 yr.	50	2848	1833	2301	2190	2733	2785
B. O'H. 76 yr.	75	30	9	103	107	44	50
J. M. 57 yr.	75	35	12	35	40	30	35

microscopic pathologic examinations, which were made by Dr. A. B. Ragins), and secondly, there was no evidence of activity of accessory adrenal glands or rests, as shown by the tests with ACTH.

The five patients were kept in a eu-adrenal state by daily therapy with cortisone acetate, and from Table I it can be seen that there was very little change in the circulating levels of eosinophils when either ACTH or placebo were injected. However, in each patient there was a significant drop in circulating eosinophils seen four hours after injecting epinephrine into them. This is shown clearly in Fig. 1. In four patients, C. G., J. M., B. O'H., and I. S., the fall in eosinophil cells was 78, 70, 66, and 54 per cent, respectively, of pre-test levels. In patient B. W. there was only a 36 per cent drop in eosinophil cells.

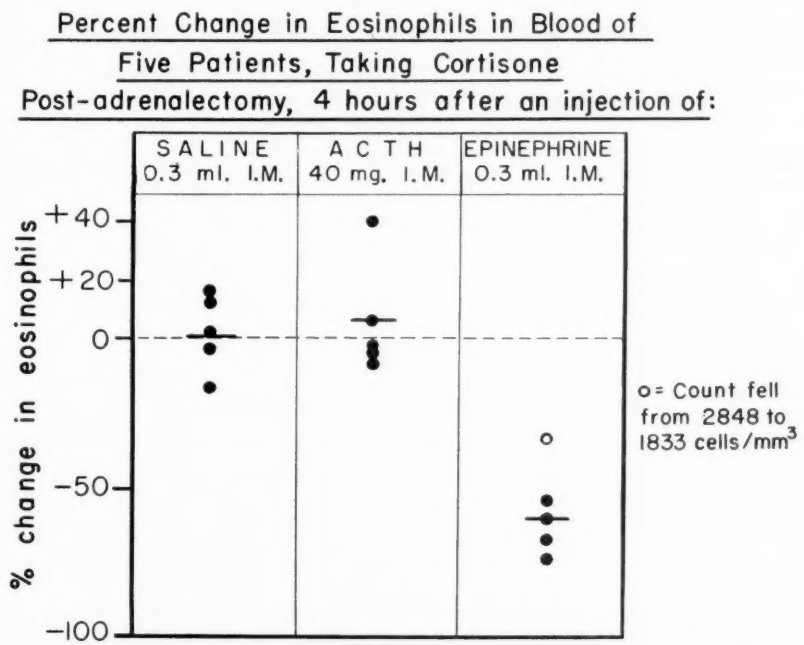

Fig. 1.—A scattergram of the per cent change in eosinophil cells in the blood of five patients with bilateral adrenalectomy (maintained in a eu-corticoid state by oral therapy with cortisone acetate) four hours after injection of 0.3 ml. saline, 40 mg. ACTH, and 0.3 ml. epinephrine. The bars indicate the mean four-hour change in eosinophil cells.

However, his eosinophil cell counts were abnormally high at the time of the study (2,800 cells per cubic millimeter) and a fall of over 1,000 cells per cubic millimeter of blood in the eosinophil count is highly significant under these conditions. The magnitude of response of the eosinophils to epinephrine in this patient corresponds to the degree of fall seen in allergic individuals, and healthy subjects with high eosinophil counts, when their normal adrenal glands are stimulated by single, 40 mg. intramuscular injections of ACTH.[3, 15]

We interpret the above data to indicate that epinephrine eosinopenia does not necessarily develop as the end result of successive stimulation of the hypothalamus, pituitary gland, and adrenal cortex, which is thought to be triggered

by an injection of epinephrine, or by liberation of epinephrine from the adrenal medulla. Epinephrine can produce eosinopenia in the absence of the adrenal, but seems to require the presence of adrenocortical hormones to be effective as an eosinopenic agent, since it is known that epinephrine does not produce eosinopenia in *untreated* Addison's disease.[6]

Unfortunately, it is not considered wise or proper, at present, to withhold daily replacement therapy with cortisone acetate from the five patients with carcinoma of the prostate reported here, and we do not know for certain if parenteral epinephrine will produce an eosinopenia in surgically induced Addison's disease in the absence of cortisone or other cortical hormones. Some observations on five patients with tuberculous or fibrotic Addison's disease may be of value. Robinson, in a personal communication to us,[18] reported that he had studied a patient with Addison's disease in whom he observed a 70 per cent fall in circulating eosinophil cells below pre-test levels, four hours after injection of epinephrine. We have repeated his observation in five patients with Addison's disease who were tested with placebo, ACTH, and epinephrine before and during therapy with cortisone. Injections of epinephrine, ACTH, or placebo had no significant effect on the circulating eosinophil cell level before treatment with cortisone, and injection of ACTH or placebo was without effect during therapy with cortisone. However, epinephrine produced a significant drop in eosinophil levels in three of the patients with Addison's disease after they had been taking cortisone by mouth, and in the other two treated patients the injection of epinephrine did not reduce the circulating eosinophil cell count. We cannot offer an explanation, at this time, which will clarify these equivocal results to parenteral injection of epinephrine into patients with Addison's disease.

The data presented here offer further evidence against the use of the four-hour eosinophil cell response to parenteral injection of epinephrine as a test of the function or integrity of the pituitary-adrenal system in man. The observations do not indicate that epinephrine may not stimulate the anterior pituitary gland to liberate ACTH in healthy individuals. This can be tested by measuring ACTH or Compound F (preferably the former) in the blood of healthy persons before and after stimulation with epinephrine. As far as we are aware, this has not been done.

SUMMARY

Five patients with carcinoma of the prostate had both their testes and adrenal glands removed, and were maintained in a eu-adrenal state by therapy with oral cortisone. Direct eosinophil counts were made before and four hours after injection of epinephrine, saline, and ACTH. There was a significant fall in eosinophils after injection of epinephrine, but ACTH or saline had no effect on the eosinophil level.

The data indicate that epinephrine eosinopenia occurs in the presence of cortisone, despite complete interruption of the pituitary-adrenal system. Because of this finding, and other observations in the literature, the four-hour eosinophil cell response to parenteral epinephrine should not be used to test the function or integrity of the pituitary-adrenal system in man.

We wish to thank Dr. John G. Vandenberg for his kindness in allowing us to study his patients.

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ADVERSE EFFECT OF CORTISONE ON MARROW REGENERATION FOLLOWING IRRADIATION

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IN A variety of diseases including rheumatic fever, rheumatoid arthritis, lupus erythematosis, and refractory anemia, cortisone[4, 10, 13, 15] may exert a stimulating effect on erythropoiesis as indicated by a granulocytic leukocytosis, thrombocytosis, reticulocytosis, and by an increase in circulating erythrocytes. This current concept of cortisone as a marrow stimulant might suggest its usefulness in combined thermal and irradiation damage. The present study was undertaken to determine whether cortisone could assist in the repair of a marrow damaged by irradiation. Under the conditions of this experiment it became evident that, far from stimulation, cortisone had a retarding effect on the regeneration of blood-forming tissues.

MATERIALS AND METHODS

Plan of Experiment.—Sprague-Dawley rats, weighing 200 to 250 Gm., were maintained on a diet of Purina. *Group 1* consisted of 35 animals given 10 mg. of cortisone acetate† intramuscularly each day. Three animals were killed every two days. None of these animals were irradiated. *Group 2* consisted of 38 rats exposed to 200 r ‡ whole body irradiation. Beginning the third day after irradiation, and continuing fourteen days, one-half of the animals were given 30,000 units of penicillin and 10 mg. of cortisone by intramuscular injection daily. The remaining nineteen control animals were injected each day with 30,000 units of penicillin alone. A single group of six animals was sacrificed on the third day following irradiation, and six animals from each group were sacrificed on the eleventh and twenty-first postirradiation days. Five days before the final groups were killed, radioiron (Fe^{55}) was injected intravenously into the animals of each group. At the time of sacrifice the radioiron content of the red cells was determined. *Group 3* consisted of 60 rats given 500 r total body irradiation§ with either cortisone and penicillin or penicillin alone on the same dosage schedule as the animals in Group 2. Six animals were sacrificed on the second postirradiation day, and six on the fourth day following irradiation. On the eleventh postirradiation day, five animals from each group were sacrificed, and at twenty-three to twenty-five days following irradiation six more animals from each group were killed. Cortisone and penicillin were administered through the eighteenth day. Radioiron was given four days before the termination of the experiment, and the level of red cell radioactivity was subsequently determined. Peripheral blood studies and marrow examinations were performed and the carcass, adrenals, spleen, and kidneys were weighed at the time of sacrifice of each animal.

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†Materials kindly supplied by Merck & Co.

‡Irradiation given with ½ Cu 1 Al filter, T-S distance 157 cm. 220 kv. and 15 Ma. Given at a rate of 15 r per minute.

§Irradiation given with ½ Cu 1 Al filter, T-S distance 157 cm. 220 kv. and 15 Ma. Given at a rate of 9 r per minute.

Methods.—Blood was drawn from the heart into a heparinized syringe for peripheral blood studies. Hemoglobin was determined by the oxyhemoglobin method on an Evelyn colorimeter. Platelet counts were done by the direct method using Rees-Ecker fluid. Bone marrow was obtained by drilling a hole in either end of the femur and expressing the marrow under pressure with oil. The marrow was then smeared and stained with Wright's and Jenner-Giemsa stains. Sections were also obtained from the sternum and femur after fixation in Vandegrift's solution. Paraffin sections of the sternal marrow were cut at 10 micra and stained with hematoxylin and eosin. The total marrow present was estimated. From previous experiences it was known that the marrow cavity in sternum and femur is filled with uniform sheets of cells filling approximately 96 per cent of the cavity.[8, 9] In Tables I and II total cellularity of the marrow is expressed as a percentage of the normal amount. Thus a figure of 25 indicates the estimated presence of 25 per cent and the absence of 75 per cent of the natural cellular marrow filling. This figure represents an approximation indicating a trend rather than an absolute measurement. Samples to be analyzed for radioactive iron content were precipitated, electroplated, and counted according to previously described techniques.[3]

RESULTS

Animals in Group 1, sacrificed while on cortisone, showed an eosinopenia (mean count 22 per cubic millimeter) with a rebound to over 200 per cubic millimeter after therapy, a slight rise of hemoglobin from an initial level of 14.0 to 15.8 Gm. per 100 ml. during the second week of therapy, and no dis-

TABLE I. 200 R TOTAL BODY IRRADIATION TO ALL RATS ON 0 DAY; PENICILLIN THERAPY TO ALL ANIMALS AFTER FOUR DAYS

THERAPY	DAY OF SACRIFICE	WEIGHT CHANGES IN GRAM	PERIPH. W.B.C.	BONE MARROW			
				TOTAL (PER CENT)	MYEL. (PER CENT)	ERYTH (PER CENT)	HISTOLOGICAL
None	4	−10	average	70	16	84	General damage to all cells. Nuclei of myeloid series are ill defined, washy in outline, many are hypersegmented or slow clumping. Cytoplasmatic granules are indistinct.
		+01	1,750	50	29	71	
		−06		70	17	83	
		−08		--	--	--	
		−10		70	29	71	
		−09		70	30	70	
Controls	11	+04	average	80	17	83	Well-defined cells and nuclei with regeneration, especially in erythroid series.
		+05	3,830	60	39	61	
		+06		70	28	72	
		+02		70	35	65	
		+01		60	53	47	
	23	+20	average	90	29	71	Well-defined cells with regeneration in myeloid and erythroid series.
		+06	5,775	95	19	81	
		+08		95	22	78	
		+08		85	30	70	
		+55		85	24	76	
		+16		90	76	24	
Cortisone	11	−29	average	50	40	60	Marrows depleted, abnormal forms of nuclei still present in myeloid series. Early regeneration of erythroid but not of myeloid series.
		−28	2,992	50	14	86	
		−16		50	32	68	
		−22		60	30	70	
		−12		60	33	67	
		−14		40	29	71	
	23	−48	average	30	23	77	Generally depleted marrows with little evidence of regeneration in the myeloid, but active regeneration in the erythroid series.
		−50	4,770	40	21	79	
		−02		55	31	69	
		−85		30	21	79	
		−68		50	34	66	

cernible effect on the total leukocyte count. Marrow studies showed an over-all decrease in cellularity of approximately 25 per cent during the period of cortisone administration. The weight of the animals decreased 10 per cent, but returned to normal within six days after cessation of therapy. Under cortisone treatment the splenic weight decreased 30 per cent and adrenals 43 per cent, while the weight of the kidneys increased 3 per cent. These data were taken to indicate that a biologically effective dosage of cortisone had been administered.

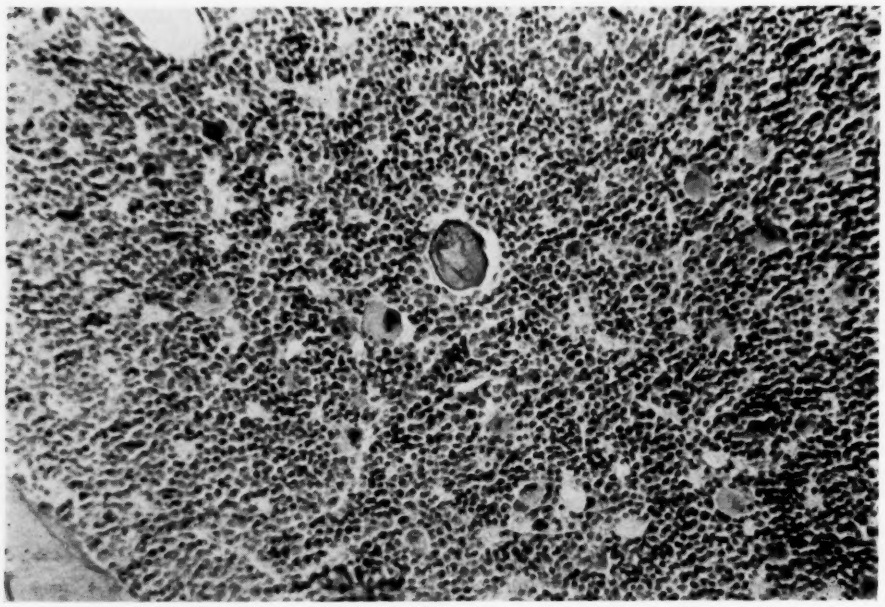

Fig. 1.—Rat bone marrow, untreated natural control.

Data on animals of Group 2 are presented in Table I. By the fourth day following 200 r total body irradiation a moderate amount of marrow damage was visible. The marrow damage appeared progressive over twenty-three days in the cortisone-treated animals, while repair was almost complete in the animals not on cortisone. The differential count of the bone marrows as well as sections indicated that the myeloid cells were more severely affected and were slower in regeneration than the erythroid series (see figure in Table I). Peripheral blood elements also were more severely depressed in the cortisone group. Radioiron uptake in the cortisone group averaged 360 counts per minute per milliliter of blood, while in the control group there were 512 counts per minute per milliliter of blood.

Data on animals in Group 3, treated with 500 r total body irradiation, are presented in Table II. In this group nine animals from the cortisone-treated group and four animals in the control group died during the experiment. While splenic and adrenal weights decreased in both groups immediately following

Fig. 2.—Rat bone marrow, seventy-two hours following 500 r total body x-radiation.

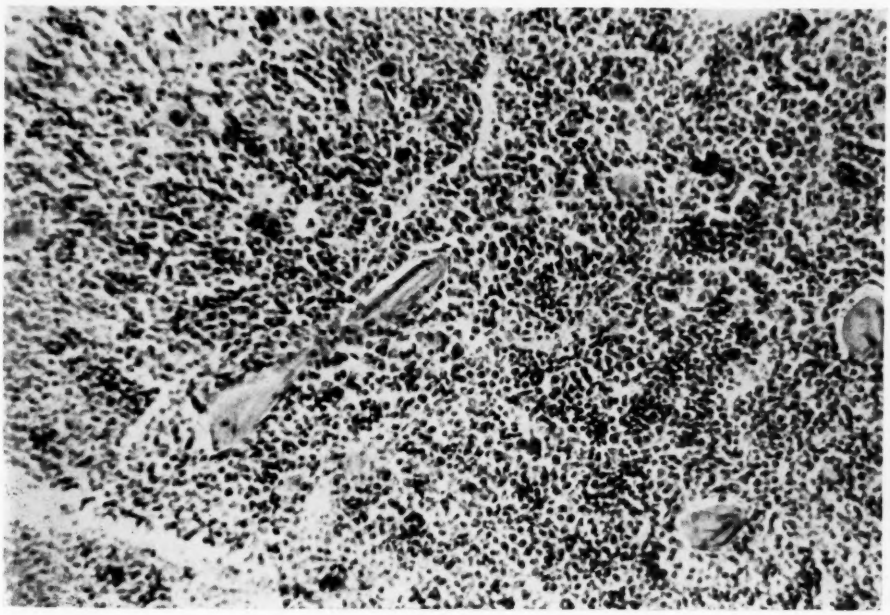

Fig. 3.—Rat bone marrow, twenty-five days following 500 r total body x-radiation.

irradiation, there was a rapid return to normal in organ weights in control animals in contrast to further weight loss in the cortisone-treated animals. Fig. 1 shows the bone marrow of an untreated rat for comparison. Figs. 2 and 3 show the bone marrow of rats at three and twenty-five days after 500 r total body x-radiation. Figs. 4 and 5 show the adverse effect of cortisone on the regeneration of marrow in x-radiated rats. For at least eleven days after irradiation there was extensive marrow destruction and marked depression of leukocytes and thrombocytes in the peripheral blood. In the control group marrow repair was nearly complete by the twenty-fifth day, although some residual leukopenia and anemia remained. In the cortisone-treated group, however, marrow was

TABLE II. 500 R TOTAL BODY IRRADIATION TO ALL ANIMALS ON 0 DAY; PENICILLIN THERAPY TO ALL ANIMALS AFTER FOUR DAYS

					BONE MARROW			
THERAPY	DAY OF SACRIFICE	WEIGHT CHANGES IN GRAM	PERIPH. W.B.C.		TOTAL (PER CENT)	MYEL. (PER CENT)	ERYTH. (PER CENT)	HISTOLOGICAL
None	2	−20	Poly	1330	50	28	72	Generally early irradiation damage to all cells with hypersegmented nuclei in myeloid series.
		+38	Lymph	9434	50	31	69	
		+33	Mono	94	100	52	48	
		+22	Eos	42	100	38	62	
		+05			100	41	59	
	4	−12	Poly	272	10	37	63	Generally severely damaged marrow with necrotic fatty areas.
		−66	Lymph	879	20	16	84	
		−18	Mono	58	10	37	63	
		−24	Eos	21	25	49	51	
		−24			15	33	67	
		−32			05	41	59	
Controls	11	+38	Poly	410	45	16	84	Nuclear definition better in myeloid series but only little evidence of regeneration. Early regeneration in erythroid series.
		+16	Lymph	1134	50	22	78	
		+ 0	Mono	52	40	10	90	
		−08	Eos	52	50	9	91	
		+44			65	23	77	
	23	+16	Poly	1266	60	32	68	Regenerating myeloid and erythroid series, in recovering marrow.
		+42	Lymph	1105	85	18	82	
		+14	Mono	90	90	55	45	
		−16	Eos	24	50	63	37	
	25	+32	Poly	1915	100	23	77	Marrow fully recovered.
		+12	Lymph	4306	100	35	65	
			Mono	29				
			Eos	--				
Cortisone	11	−60	Poly	777	50	--	--	Degenerating marrow with fatty replacement. Nuclei indistinct with clumping chromatin. Differential count responsible in two cases.
		−52	Lymph	524	40	19	81	
		−72	Mono	12	40	--	--	
		−82	Eos	12	25	17	83	
		−96				19	81	
	23	−44	Poly	1266	50	30	70	Better nuclear definition now in myeloid series. Early foci of regeneration in erythroid series.
		−124	Lymph	1105	20	27	73	
		−42	Mono	90	50	29	71	
		−72	Eos	24	50	29	71	
	25	−40	Poly	2222	50	15	85	Regenerating marrow especially erythroid series. Good nuclear definition now also in myeloid series.
		−10	Lymph	1382	50	28	72	
			Mono	29				

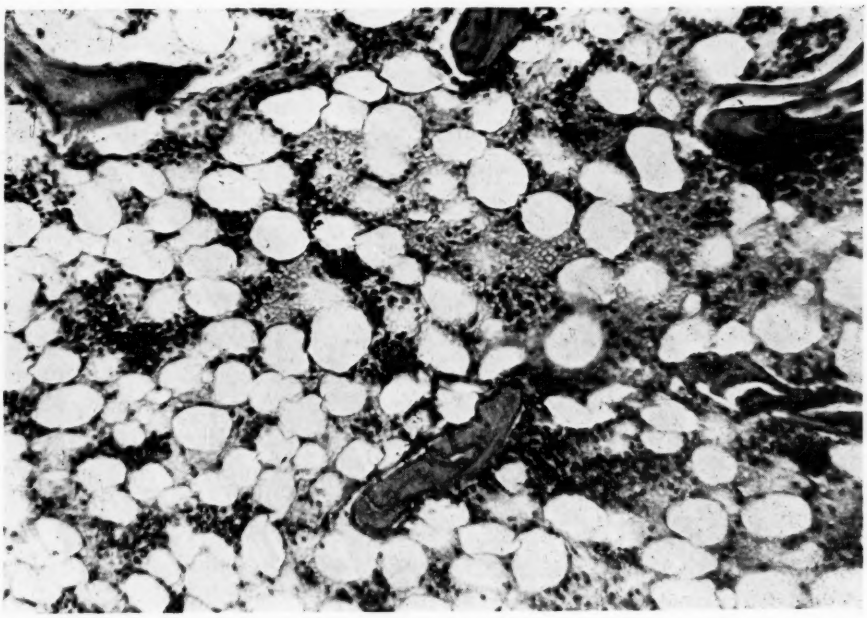

Fig. 4.—Rat bone marrow, eleven days following 500 r total body x-radiation with cortisone therapy.

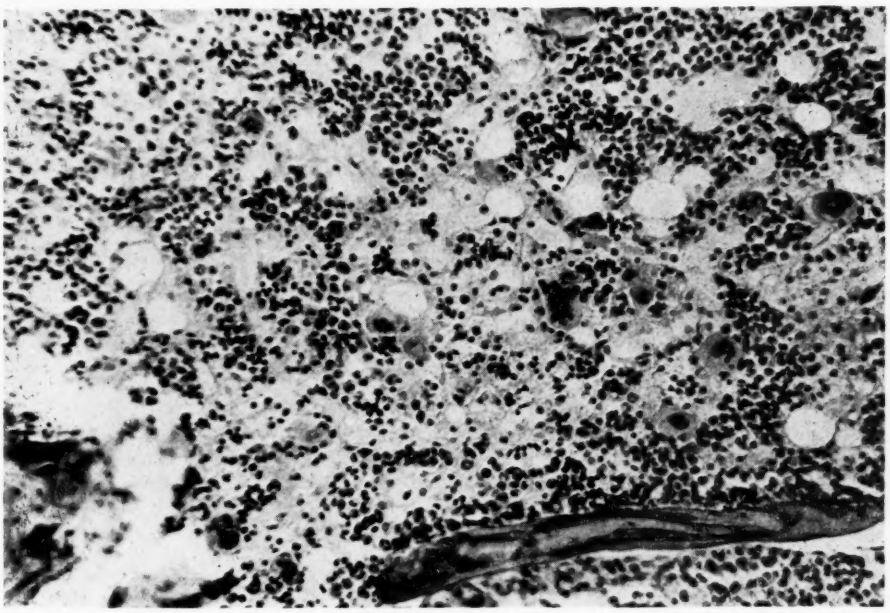

Fig. 5.—Rat bone marrow, twenty-five days following 500 r total body x-radiation with cortisone therapy.

still only one-half normal in cellularity twenty-five days after irradiation. The depression of myelopoesis and the more prolonged damage in the myelocytic as compared with erythroid tissue was even more marked than in Group 2 (see Table II). The hemoglobin concentration, platelet count, and total white blood count in the cortisone-treated animals were lower than in the control group, while the absolute granulocyte count did not differ significantly (Table II). Radioiron uptake in the blood of cortisone-treated animals averaged 390 counts per minute per milliliter as compared with 540 counts per minute per milliliter in the control group.

DISCUSSION

The effect of cortisone on normal rats included loss of total body weight, decrease in size of spleen and adrenal glands, eosinopenia, and a mild depression of the bone marrow. X-radiation alone resulted in weight loss, marrow damage, followed by repair over a period of three to four weeks. The administration of cortisone to irradiated rats resulted in a cumulative harmful effect of irradiation plus that of the hormone. These animals suffered a more severe weight loss and blood and bone marrow depression than was found with either agent alone. Histologic sections showed a strikingly slower rate of marrow regeneration, and radioiron uptake studies indicated a lower level of erythropoiesis in the cortisone-treated animals. In these experiments the time sequence of cortisone therapy after irradiation was planned to simulate the clinical situation following a mass discharge of nuclear energy and irradiation sickness. Here flash burns might be an added incentive to oxysteroid therapy. Previous reports of other workers[5, 11, 12, 14] showed that cortisone given prior to or immediately following x-ray radiation had little effect on the severity of irradiation damage. The present observation, wherein cortisone was administered after irradiation damage was established, demonstrated the hormone to be detrimental to recovery.

The mechanism of this cortisone effect is not known. Both depletion of sulfhydryl groups[1, 2] and injury to lymphoid tissue[6] may be of importance in potentiating irradiation damage. While cortisone may produce both these effects, cortisone therapy in these studies was administered several days after irradiation, at a time when the previously mentioned mechanisms have not been demonstrated to be effective.

CONCLUSION

Cortisone administered following irradiation in rats impairs the recovery of hematopietic tissues.

The authors would like to express their appreciation to Dr. Templeton for his assistance in irradiation of the animals.

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THE FREQUENCY OF LABORATORY INFECTIONS WITH HISTOPLASMA CAPSULATUM: THEIR CLINICAL AND X-RAY CHARACTERISTICS

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INTRODUCTION

IN ANY laboratory in which infectious materials are studied and handled, the possibility of infection of laboratory personnel with the agents must be considered. Such laboratory infections are known to occur with bacteria, rickettsiae, viruses, and fungi.[1] The present paper presents an attempt to assay the laboratory hazard associated with the handling of a pathogenic fungus, *Histoplasma capsulatum*, the clinical illness which accompanies such laboratory infection, and the characteristics of the chest lesions which appear in the x-ray.

MATERIAL AND METHODS

A total of fifty-six persons has been employed at the Mycoses Laboratory of the Public Health Service in Kansas City, Kansas, during its six and one-half years of existence. Ever since the laboratory was established, it has been routine for each new employee, upon entering on duty, to receive tuberculin and histoplasmin skin tests and a chest x-ray; those negative to the skin tests have been retested at intervals of three to six months during their employment. All personnel have been x-rayed at intervals of three to six months regardless of their skin reactions. Persons whose skin tests were originally negative and changed to positive during their employment were considered to have become infected, and were observed closely for clinical or x-ray evidence of this infection. Most of the personnel have had more or less intimate contact with the laboratory work, and hence, exposure to possible laboratory infection with the fungi which are being studied, in particular *H. capsulatum*.

RESULTS

Of the 56 employees, 30 (54 per cent) were positive to histoplasmin when first tested. The remaining 26 were skin-test negative at first test. Of those, four had only one test, and five others remained negative for periods of three, three, eight, sixteen, and twenty-eight months, respectively. The remaining 17 persons developed positive histoplasmin skin tests after periods of employment varying from one month to three years, averaging approximately 4.2 months. The average age of these persons was 29.3 years. The details of the skin test observations in the 17 converters and the 5 nonconverters are seen in Table I.

From this table it seems that the total susceptible months of observation in these 22 persons was 128.50. The conversion rate per 100 susceptible person months is calculated as follows: $\frac{17}{128.5} \times 100 = 13.23$ per cent per month.

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Since the laboratory is situated in an endemic area of histoplasmosis where the infection rates are high,[2] the laboratory personnel also would be exposed to this natural source of infection. Therefore, determining the conversion rate (change from negative to positive skin test) of persons living in this area, but not employed in the laboratory, would be essential in appraising the significance of the conversion rate for the laboratory personnel.

For this purpose there were available the records of 5,689 Kansas City school children who were being followed by repeated skin tests and x-rays.[3] The records of this survey were analyzed by using experience-table methods, and the results are shown in Table II.

The over-all conversion rate for the group of school children was 0.47 conversions per 100 susceptible person months. For the laboratory personnel the rate was 13.23 conversions per 100 susceptible person months. A chi-square test of the significance of the difference between these rates indicates that there is less than one chance in a thousand that a difference as great as the one observed could arise by chance. (Chi-square = 342, 1 degree of freedom).

Clinical and X-ray Characteristics of Laboratory Infections.—Of the seventeen persons who converted while working in the laboratory, seven gave histories of definite illnesses of an influenza-like nature which occurred about the

TABLE I. TIME REQUIRED FOR CHANGE IN SKIN TESTS AMONG 17 LABORATORY PERSONNEL WHOSE HISTOPLASMIN SKIN TEST CHANGED FROM NEGATIVE TO POSITIVE (CONVERTERS) DURING EMPLOYMENT. ALSO SHOWN ARE RECORDS OF FIVE LABORATORY PERSONNEL WHOSE SKIN TESTS REMAINED CONSISTENTLY NEGATIVE

CONVERTERS			
	PERIOD FOR CONVERSION (IN MONTHS)		
CASE NO.	MINIMUM	MAXIMUM	AVERAGE
1	0	2	1
2	0	1.5	0.75
3	2.5	3	2.75
4	7	8.5	7.75
5	0	3	1.5
6	1.5	4.5	3
7	1.5	4.5	3
8	0	2	1
9	1.5	4.5	3
10	4	8.5	6.25
11	35	38	36.5
12	0	1	0.5
13	0	1	0.5
14	0	1	0.5
15	0.5	1.5	1
16	0.5	2	1.25
17	0.5	1.0	0.75
Total			71.00
NONCONVERTERS			
CASE NO.	PERIOD SKIN TEST REMAINED NEGATIVE (IN MONTHS)		
1	3.5		
2	2.5		
3	8.0		
4	15.75		
5	27.75		
Total	57.50		
Total susceptible person months			128.50

TABLE II. CALCULATED HISTOPLASMIN SKIN TEST CONVERSION RATES FOR KANSAS CITY SCHOOL CHILDREN 1946-1949*

TEST PERIOD NUMBER X	TIME INTERVAL (MONTHS) M	NUMBER NEGATIVE AT START OF PERIOD† N	NUMBER WITHDRAWN DURING PERIOD W	NUMBER CONVERTING TO POSITIVE DURING PERIOD D	AVERAGE NUMBER OF SUSCEPTIBLES DURING PERIOD‡ S	NUMBER OF SUSCEPTIBLE PERSON MONTHS DURING PERIOD SM	NUMBER OF NEW NEGATIVES ENTERING FOR NEXT PERIOD E	CONVERSION RATE PER 100 SUSCEPTIBLE PERSON MONTHS 100 D/SM
1	4	1814	247	56	1539.0	6156.0	16	0.91
2	3	1527	133	20	1384.0	4152.0	139	0.48
3	5	1513	608	20	895.0	4475.0	977	0.45
4	4	1862	219	22	1632.0	6528.0	1	0.34
5	3	1622	218	35	1386.5	4159.5	107	0.84
6	5	1476	484	18	983.0	4915.0	1036	0.37
7	7	2010	369	26	1628.0	11396.0	----	0.23
Over-all				197		41781.5		0.47

*Method used is similar to life table analysis.[6]

†$N_x - 1 = N_x - W_x - D_x + E_x$

‡$S_x = N_x - W_x - \frac{1D}{2} x$

time their skin tests changed from negative to positive (Table III). These illnesses usually lasted from three to ten days, confining the patient to bed with cough, fever, and loss of weight. In the most severe case, the patient was absent from work for nine weeks. Since the illness in this case was carefully observed, the details are of interest. Her illness began approximately one month after entering employment. The onset was gradual, with malaise and headache followed by cough and slight chest pain. There was a daily temperature elevation from 100 to 101° F. After several days the chest pain disappeared. Headache, cough, malaise, weakness, and temperature continued. No changes were visible in the chest roentgenograms during the illness, but a pneumonic lesion appeared in the right third and fourth interspaces three months after recovery. The sedimentation rate was found elevated (25 mm. in sixty minutes) on the eighteenth day of illness. It was still slightly elevated (12. mm. in sixty minutes) when last tested 351 days after onset. Results furnished strong supporting evidence that the infection was caused by *H. capsulatum*.

Five of the seven persons with illnesses had positive x-ray findings. One of these had an ill-defined basilar pneumonic lesion in the left lower lobe and a small pneumonic lesion in the right first interspace. These lesions showed contraction during fourteen months of follow-up. The second had two small nodular

TABLE III. SUMMARY OF CLINICAL AND X-RAY OBSERVATIONS ON 17 LABORATORY PERSONNEL WHOSE HISTOPLASMIN SKIN TEST CHANGED FROM NEGATIVE TO POSITIVE (CONVERTERS) DURING EMPLOYMENT. SIMILAR OBSERVATIONS ON 5 NONCONVERTERS AMONG LABORATORY PERSONNEL ARE SHOWN

CASE NO.	AGE	CLIN. HIST. OF ILLNESS	X-RAY LESIONS	TYPE OF X-RAY LESIONS	LENGTH OF X-RAY OBSERVATION (MONTHS)	FATE OF LESIONS
CONVERTERS						
1	28	0	0		20	
2	22	+	+	Nodular-2	23	Scar
3	29	+	+	Pneumonic-2	19	Contracting
4	32	+	0		18	
5	40	0	0		0	
6	27	+	0		8	
7	30	0	0		64	
8	45	0	0		34	
9	25	0	0		2	
10	26	0	+	Mediastinal Node	70	Regression
11	25	0	0		41	
12	23	0	+	Pneumonic	1	
13	22	+	+	Pneumonic	4	Regressing
14	23	0	0		18	
15	48	+	+	Pneumonic	10	
16	26	0	0		8	
17	28	+	+	Pneumonic	2	
Average	29.3				20.1	
NONCONVERTERS						
1	22	0	0		3	
2	24	0	0		6	
3	27	0	On 1st film	Arrested Min. Tuberculosis Several Years	9	No change
4	38	0	On 1st film	Thorocoplasty Sev. Years Previous	15	No change
5	31	0	0		27	

lesions in the right fourth and left fourth interspaces which healed by fibrosis after nineteenth months. The third person showed an enlarged, right mediastinal node which regressed after fourteen months observation. The fourth developed a pneumonic lesion in the right third and fourth interspaces which appeared after the clinical illness had subsided, and persisted for at least four months. The fifth showed a pneumonic lesion in the right fourth interspace which cleared in several weeks and a pneumonic lesion with enlarged hilar lymph nodes on the left which persisted for at least six weeks. Two other persons showed x-ray lesions without accompanying illness; one a pneumonic lesion in the left fourth interspace which has been followed for less than a month, the other a pneumonic lesion in the left third interspace which showed regression after four months of follow-up. The average follow-up period by x-ray for the group was 20.1 months, but five of the converters were followed by x-ray for less than six months (for details of these 17 converters, see Table III).

It is to be regretted that sufficient blood samples were not secured on these persons to evaluate adequately the serological changes accompanying infection. In only three cases (Cases 15, 16, and 17) were sufficient samples obtained to get a fairly complete picture. No serological changes were detected in Case 16. Both of the other cases developed positive precipitin tests which fell toward normal with recovery. One (Case 17) also developed a low titer complement fixation test. Both of these persons had clinical illness. The serological changes will be reported in a later publication.

Most of the converters had little or no sputum with their illness, and specimens for culture were secured in only two cases. None of these specimens were positive for *H. capsulatum* by culture or animal inoculation.

DISCUSSION

It is apparent from Table II that the risk of infection with *H. capsulatum* while working in the laboratory is very much greater than among the general population of the same city. In fact, the risk of laboratory infection was 13.23/0.47, or approximately 26 times greater than the risk among the school children. Since both groups are similar, as far as can be determined, except for age and occupation, the higher conversion rate in the laboratory personnel may be due to: (1) decreased resistance arising from their greater age, or (2) increased exposure to *H. capsulatum* in the laboratory, or (3) a combination of these two factors. Sufficient data to determine effects of age on susceptibility to infection with *H. capsulatum* at ages beyond 16 years are not available. However, there is no data which suggests that susceptibility of adults to this disease is greater than that of children. Therefore, it seems probable that working in a mycological laboratory with *H. capsulatum* constitutes an increased hazard of contracting an infection with this fungus. These results are similar to those reported[4] for persons employed in a laboratory studying *Coccidioides immitis*.

The history of clinical illness accompanying conversion to the histoplasmin skin test was high (41 per cent). It is interesting to compare this incidence of illness with that reported by Smith and associates[5] for converters to the coccidioidin skin test among Army personnel. Among coccidioidin converters, 24

per cent showed a definite illness or other diagnosible change, while another 16 per cent showed mild illness but were not diagnosed (so-called "missed and subclinical infections"). It is interesting to note, however, that the percentage showing no determinable illness among his group (59.4 per cent) is the same as that reported for the present group (59 per cent).

The serological changes accompanying infection in two of the three cases in which sufficient satisfactory samples were obtained are striking, and substantiate the usefulness of the serological tests in diagnosing the infection.

SUMMARY

The conversion rate from negative to positive skin test among laboratory employees was 13.23 conversions per 100 susceptible person months, while in a large group of school children, the conversion rate was 0.47 conversions per 100 susceptible person months. The difference between these rates is statistically significant and suggests that employment in the laboratory increased exposure to infection with *H. capsulatum*.

This conversion in skin test (change from negative to positive) was accompanied by a definite clinical illness in 7 of the 17 laboratory personnel under observation (41 per cent). This illness was nonspecific in character, but in one instance caused nine weeks loss from work. Five of these 7 persons with illness accompanying conversion also showed x-ray changes in their lungs at the same time.

Seven of the 17 converters (41 per cent) had x-ray changes appear in their lungs about the time of conversion which regressed slowly. The lesions which appeared were pneumonic in character in five persons, nodular in one, and involved the lymph nodes only in one person. Single lesions appeared in the x-rays of four persons, two lesions in each of the remaining three persons.

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BLOOD PLASMA PEPSINOGEN

II. The Activity of the Plasma from "Normal" Subjects, Patients with Duodenal Ulcer, and Patients with Pernicious Anemia

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PATIENTS with duodenal ulcer have a greater rate of pepsinogen excretion in the urine (uropepsin) than do persons without any gastrointestinal disturbances.[1] Conversely, the pepsinogen excretion by patients with pernicious anemia is negligible.[1, 2] Since the proteolytic activity of plasma at pH 1.5 is due principally to the autocatalytic conversion of pepsinogen to pepsin,[3] it became pertinent to determine the activity of plasma from subjects with and without known dysfunction of the gastric mucosa.

METHOD

The studies reported herein were performed during the period from December, 1949, to March, 1950, in order to obviate any influence of seasonal factors on gastric secretion.[4]

The major criterion used in the selection of subjects was the presence or absence of gastric dysfunction. Consequently, individuals without any evidence of gastrointestinal disturbance or of any condition known to affect gastric secretion were selected to comprise the "control" group. Since hepatic cirrhosis,[4] diabetes mellitus,[4] nephritis,[4] rheumatoid arthritis,[4] osteoarthritis,[4] pregnancy,[5] hyperthyroidism,[6] and hypochromic anemia[7] have been alleged to affect gastric secretion, care was taken to exclude such states from the "control" group. The subjects comprising the duodenal ulcer group were selected on the basis of unequivocal roentgenologic or gastroscopic demonstration of the lesion at some time prior to this study. The subjects comprising the pernicious anemia group were selected on the basis of the demonstration of typical hematologic changes in the past which were corrected with liver therapy. Since the subjects were all on liver therapy, no effort was made to validate the diagnosis.

A modification of Anson's procedure[8] was utilized for the assay of the proteolytic activity of plasma at pH 1.5.[3] Although a small proportion of the activity obtained with this procedure is due to some system other than pepsin, the major proportion of the total activity is due to the autocatalytic conversion of pepsinogen to pepsin.[3] Consequently, the total proteolytic activity of the plasma, expressed as micrograms of "tyrosine" released by the action of 1 ml. of plasma at pH 1.5 in the standard assay procedure, was utilized as an index of the concentration of pepsinogen in the native plasma. For the sake of convenience, the total activity will be referred to as "pepsinogen."

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RESULTS

Constancy of Plasma Pepsinogen.—In order to evaluate the significance of the pepsinogen content of the plasma in various groups of subjects, it became essential to determine the variability from hour to hour and day to day. Accordingly, blood samples were drawn from a group of men at intervals of from two to four hours throughout the day. The plasma pepsinogen activity of the different subjects varied from 280 to 788, while the coefficient of variation of each subject's samples ranged from 2 to 22 per cent, with a mean coefficient of 8 per cent (Table I).

TABLE I. VARIATIONS IN PLASMA PEPSINOGEN OF BLOOD TAKEN AT FROM TWO TO FOUR HOURLY INTERVALS DURING THE DAY

SUBJECT	NO. OF SAMPLES	MEAN ± S.D.*	COEFFICIENT OF VARIATION (PER CENT)
1	9	572 ± 66.6	11.6
2	7	606 ± 87.6	14.5
3	8	559 ± 88.8	15.9
4	5	686 ± 75.2	11.0
5	7	639 ± 32.3	5.1
6	4	387 ± 86.3	22.3
7	8	689 ± 26.3	3.8
8	7	411 ± 30.5	7.4
9	5	751 ± 27.7	3.7
10	9	602 ± 38.4	6.4
11	8	673 ± 29.0	4.3
12	7	393 ± 30.0	7.6
13	5	649 ± 38.1	5.9
14	9	477 ± 44.3	9.3
15	9	512 ± 11.0	2.1
16	8	507 ± 37.6	7.4
17	9	280 ± 22.4	8.0
18	7	788 ± 68.5	8.7
19	7	747 ± 23.6	3.2

*Expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma in the standard assay.

To test the degree of fluctuation from day to day, the plasma pepsinogen activity of two healthy men and two healthy women was determined daily for approximately one month (Table II). The coefficient of variation was essentially the same for each subject, viz., from 12 to 18 per cent.

TABLE II. DAILY VARIATIONS IN PLASMA PEPSINOGEN

SUBJECT	SEX	DAYS	ACTIVITY MEAN ± S.D.*	COEFFICIENT OF VARIATION (PER CENT)
S. M.	M	32	590 ± 70	12
E. W.	F	31	540 ± 95	18
G. P.	F	32	500 ± 79	16
P. F.	M	32	540 ± 80	15

*Expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma in the standard assay.

When the cumulative percentage frequency of a variable is plotted on probability paper against the units of that variable, a straight line is obtained when the distribution is normal. Illustrative is the plot in Fig. 1 of the daily

values for plasma pepsinogen of a subject studied for thirty-two days, and which demonstrates that the data are arranged about a mean in the form of a normal distribution curve. It can be demonstrated, likewise, that the fluctuations from hour to hour are also random in that the values can be arranged about some mean in the form of a normal distribution curve. Accordingly, it is permissible to utilize a single sample of plasma drawn at any time of the day to characterize the individual and for comparison between individuals.

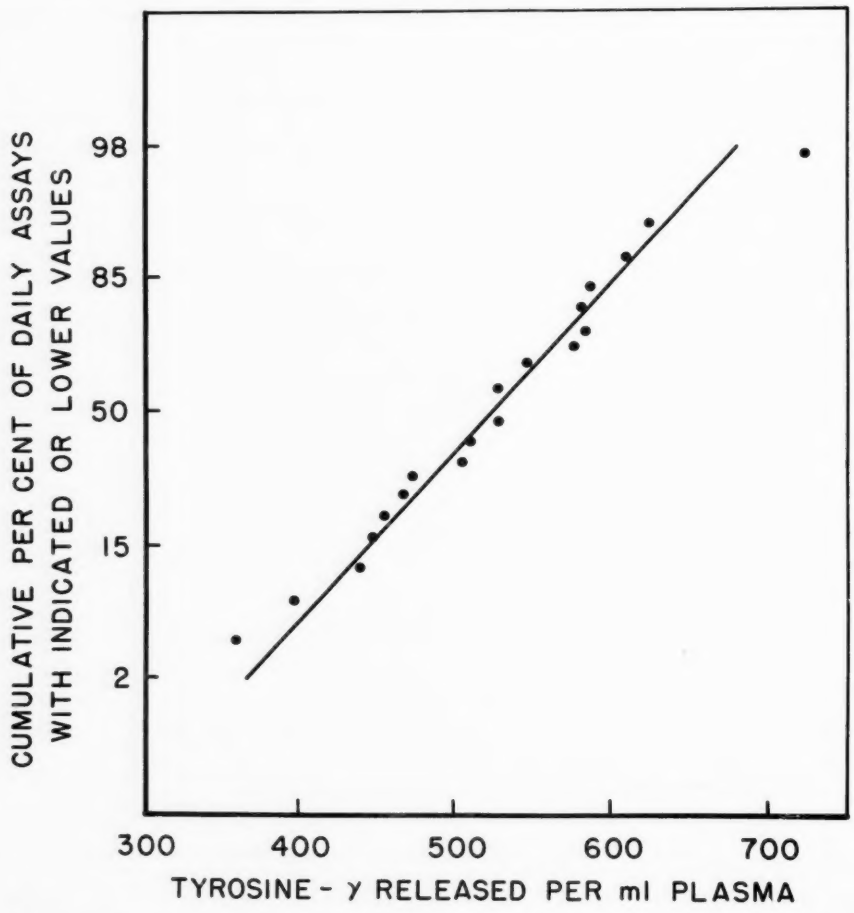

Fig. 1.—Frequency distribution of 32 daily assays of plasma pepsinogen of a healthy man.

Effect of Food.—Although previous studies on the rate of excretion of urinary pepsinogen (uropepsin) revealed no influence of food,[9] nevertheless, it was pertinent to determine the effect of eating on the pepsinogen of the plasma. Blood samples were drawn from healthy subjects at intervals of from thirty to sixty minutes for from three to four hours before and after they were given a meal consisting of isocaloric quantities of carbohydrate or fat or protein, or a mixture of the three. In no instance did the food produce a sig-

nificant effect on the plasma pepsinogen (Fig. 2). Further, dogs fasted for six days showed no significant changes in the plasma pepsinogen. In view of these observations, blood samples were drawn from the various groups of subjects approximately two hours after breakfast.

Control Group.—Assays of plasma pepsinogen were performed on 161 male and 151 female subjects of from less than 1 year to 89 years of age (Fig. 3). A summary of data reveals that the activity of the plasma increases with age until the third decade, and remains fairly constant thereafter (Table III). Although there seems to be a tendency for the values to be higher in men than women, analysis of covariance revealed no significant difference between the sexes of each age group except for the 70- to 79-year-old group.

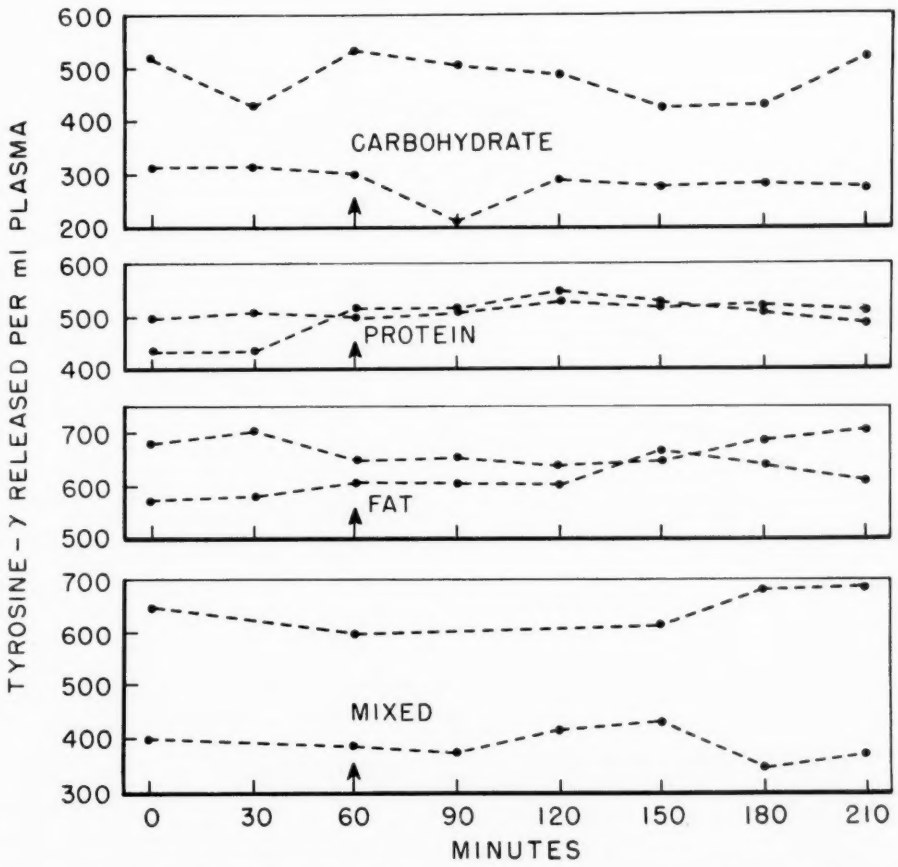

Fig. 2.—The effect of carbohydrate, fat, protein, and a mixed diet on the plasma pepsinogen.

Duodenal Ulcer.—The duodenal ulcer group consisted of ninety-eight subjects, seventeen of which were women. Unequivocal evidence of an active duodenal ulcer was demonstrated within forty months prior to the present study in eighty-eight of the patients. In eighty-four subjects, less than twenty

TABLE III. PLASMA PEPSINOGEN OF SUBJECTS WITHOUT GASTROINTESTINAL DISTURBANCES

AGE GROUPS	MEN		WOMEN		SIGNIFICANCE OF DIFFERENCE
	NO.	ACTIVITY* MEAN ± S.D.	NO.	ACTIVITY* MEAN ± S.D.	P
0-9	18	391 ± 26.1	19	449 ± 26.1	0.1-0.2
10-19	13	521 ± 39.9	22	471 ± 22.6	0.2-0.3
20-29	27	634 ± 35.1	18	571 ± 24.9	0.1-0.2
30-39	15	623 ± 43.6	17	615 ± 38.2	0.8-0.9
40-49	16	639 ± 57.0	17	630 ± 32.7	0.8-0.9
50-59	16	648 ± 59.3	20	593 ± 31.4	0.4-
60-69	15	596 ± 66.7	14	686 ± 75.6	0.4
70-79	25	749 ± 51.3	13	577 ± 33.6	0.02-0.05
80-89	16	731 ± 49.3	11	643 ± 61.2	0.2-0.3

*Expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma in the standard assay.

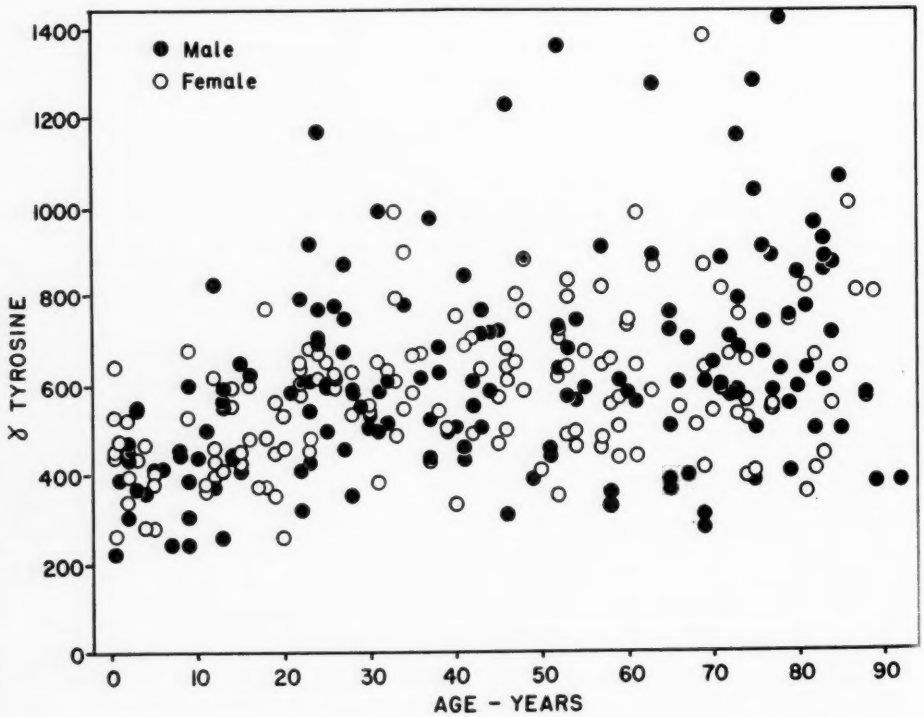

Fig. 3.—Plasma pepsinogen of subjects without gastrointestinal disorders.

months had elapsed since the activity of the ulcer had been demonstrated, while in seventy subjects, less than ten months had elapsed. The scatter of the data obtained in the ninety-eight patients is illustrated in Fig. 4.

Since the sex of the subject does not affect the plasma pepsinogen, and since the number of women in the group was too small to permit for adequate statistical analysis, both men and women with duodenal ulcer were compared with both men and women comprising the control group. It is apparent from Table IV that at all age periods, the patients with ulcer have a significantly

TABLE IV. PLASMA PEPSINOGEN IN PATIENTS WITH DUODENAL ULCER

AGE GROUPS	CONTROLS		DUODENAL ULCER		SIGNIFICANCE OF DIFFERENCE
	NO.	ACTIVITY* MEAN ± S.D.	NO.	ACTIVITY* MEAN ± S.D.	P
0-9	37	421 ± 18.2	--	----------	----------
10-19	35	489 ± 20.5	1	936	----------
20-29	45	609 ± 22.6	14	761 ± 44.8	0.001-0.01
30-39	32	619 ± 28.4	18	743 ± 48.4	0.02 -0.05
40-49	33	634 ± 31.8	23	845 ± 50.2	Less than 0.001
50-59	36	617 ± 31.4	20	956 ± 82.2	Less than 0.001
60-69	29	639 ± 50.1	15	1094 ± 104.9	Less than 0.001
70-79	38	690 ± 37.8	7	975 ± 157.6	0.001-0.01
80-89	27	695 ± 38.6	--	----------	----------

*Expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma in the standard assay.

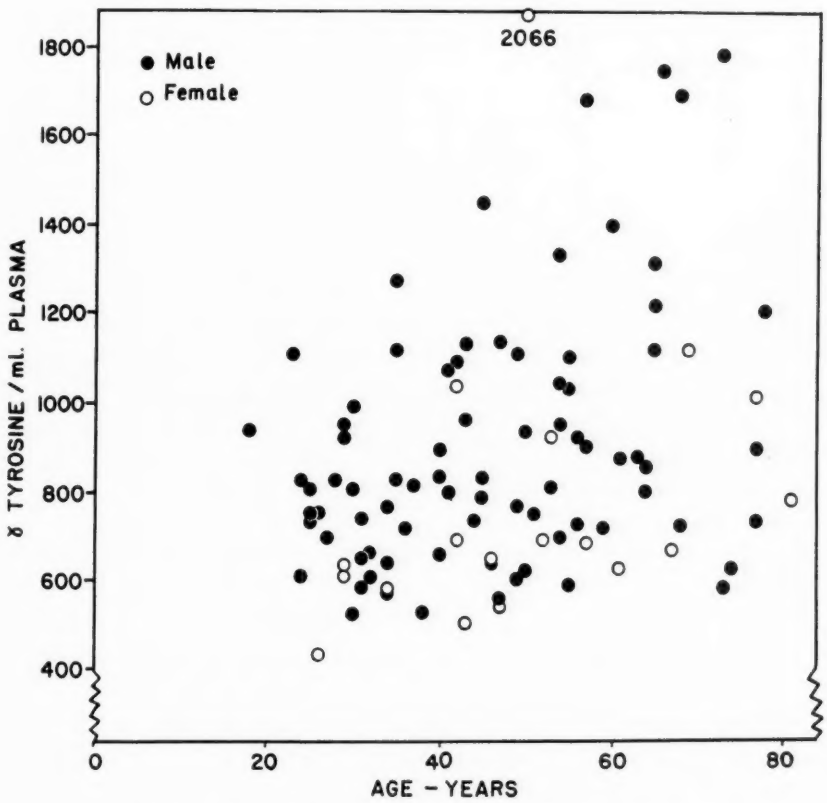

Fig. 4.—Plasma pepsinogen of patients with duodenal ulcer.

higher plasma pepsinogen activity than the control group. Further, whereas the mean values for the control group are relatively constant after the third decade, the patients with duodenal ulcer have an increasing level with increasing age.

TABLE V. PLASMA PEPSINOGEN IN PATIENTS WITH PERNICIOUS ANEMIA

AGE GROUPS	SUBJECTS WITH PERNICIOUS ANEMIA		SIGNIFICANCE OF DIFFERENCE†
	NO.	ACTIVITY* MEAN ± S.D.	P
30-39	4	205 ± 23.8	Less than 0.001
40-49	15	252 ± 25.1	Less than 0.001
50-59	19	260 ± 21.6	Less than 0.001
60-69	31	253 ± 16.1	Less than 0.001
70-79	26	258 ± 17.2	Less than 0.001
80-89	6	197 ± 19.1	Less than 0.001

*Expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma in the standard assay.

†Compared with control group.

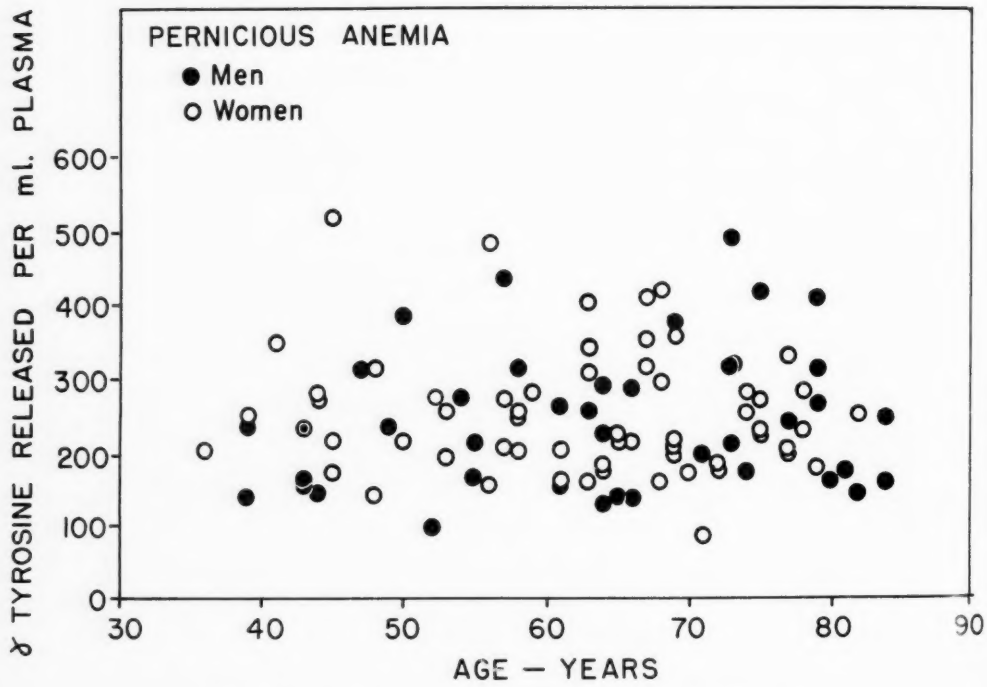

Fig. 5.—Plasma pepsinogen of patients with pernicious anemia.

*Pernicious Anemia**.—The plasma of thirty-seven men and sixty-four women with pernicious anemia was assayed for pepsinogen (Fig. 5). The values obtained at all periods were well below those of the subjects comprising the control group (Table V). Since it has been demonstrated that from 150 to 200 micrograms of "tyrosine" released by the action of 1 ml. of plasma in the standard procedure is not due to pepsinogen,[3] it is probable that the values obtained in the patients with pernicious anemia are due to minimal amounts of pepsinogen.

*We are indebted to Dr. David M. Allweiss who provided us with many samples from patients with pernicious anemia.

DISCUSSION

In a previous communication,[9] it was demonstrated that healthy men excrete appreciable quantities of pepsinogen at a fairly constant rate throughout the day and from day to day. It was noted also that the rate of excretion is not affected by ordinary activity nor by the ingestion of food. The present study reveals the reason for the aforementioned, since the pepsinogen content of the plasma is relatively constant from hour to hour and day to day and is

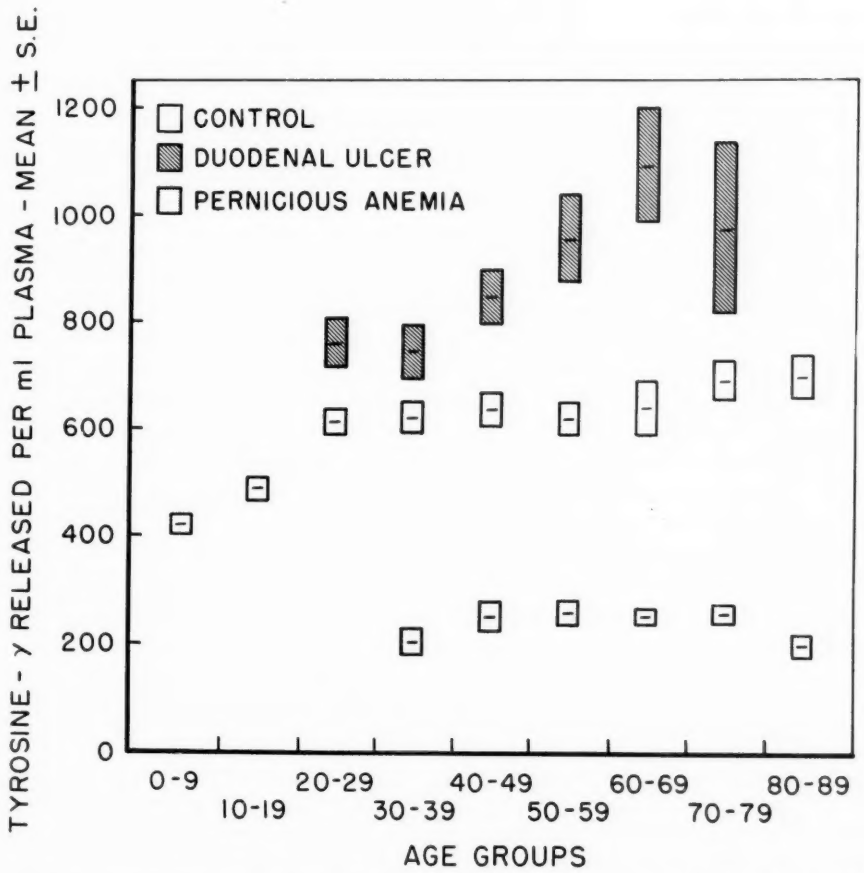

Fig. 6.—Comparison of the content of pepsinogen in the plasma of subjects without any gastrointestinal disorder, patients with duodenal ulcer, and patients with pernicious anemia. Each box represents the mean ± S.E. for each age group.

not affected appreciably by the ingestion of carbohydrate, fat, protein, or a mixed diet. It is quite possible, however, that the prolonged administration of some specific diet may affect the secretion of pepsinogen.[10]

The demonstration that the plasma pepsinogen activity increases from infancy to 20 years of age is in accord with the observations of Vanzant and associates[11] who found a similar relationship between the age of the subject and

the quantity of "free acid" in gastric juice. The relative constancy of plasma pepsinogen after the third decade is in accord also with similar findings with respect to the relation of gastric secretion and age.

Although the concentration of pepsinogen in the plasma of men tends to be higher than that of women, the difference between the sexes is statistically insignificant except for the subjects comprising the 70- to 79-year-old groups. These observations are not in accord with those of Osterberg and associates[12] who found a significantly greater peptic activity in the gastric juice of men than of women, and of Vanzant and associates[11] who found a similar relationship for the "free acid" of gastric juice. It is noteworthy that both groups of investigators excluded all data from persons with achlorhydria. The validity for eliminating such data may be questioned in the light of more recent concepts concerning the criteria for achlorhydria.[13] In the present study, no data were excluded and, consequently, subjects with low values for plasma pepsinogen may be responsible for the absence of a significant difference between the sexes.

In accord with previous observations on the excretion of pepsinogen by patients with duodenal ulcer and with the observations of Gottlieb,[14] the plasma of patients with duodenal ulcer has greater activity at acid reactions than does the plasma of normal subjects. The differences noted in the plasma pepsinogen activity of patients with duodenal ulcer, as contrasted with that of the subjects comprising the control group, are statistically highly significant at all ages (Fig. 6). For practically all age groups, the probability is less than one in a thousand that the observed differences could be due to chance grouping of the subjects (Table IV). The degree of overlapping of the data of the groups with and without peptic ulcer makes it impossible to utilize the plasma pepsinogen to determine whether or not any particular subject definitely belongs to one or the other group. This limitation, however, is no greater than that involved in evaluating the data obtained by direct examination of the volume, acidity, or peptic activity of gastric juice.[15]

Analysis of the frequency distribution of the plasma pepsinogen values of subjects aged 20 years and above revealed that the mean plasma pepsinogen, expressed as micrograms of "tyrosine" released by the activity of 1 ml. plasma, was 610 for the control group and 832 for the group with duodenal ulcer. The plasma pepsinogen values for 87 per cent of the patients with duodenal ulcer were above the mean for the whole control group, while 14 per cent of the values of the control group were above the mean of the patients with duodenal ulcer. Further, 2 per cent of the plasma pepsinogen values of the subjects in the control group were beyond one standard deviation of the mean of the group with peptic ulcer, while the values of 50 per cent of the ulcer group were beyond one standard deviation of the mean of the normal group.

The differences in the plasma pepsinogen concentrations between groups of subjects with and without duodenal ulcer become more striking when the comparison is made between subjects of the same age periods. Thus, analysis of the frequency distribution of the values at different age periods reveals

that from 76 to 100 per cent (mean = 87.3 per cent) of the patients with ulcer have values which are above the means of the respective age groups without ulcer; while from 4 to 13 per cent of the control group have values which are above the means for the groups without ulcer.

The aforementioned differences between the groups with and without duodenal ulcer are consistent with the general consensus that the patient with duodenal ulcer tends to secrete more gastric juice than the normal subject.[15] In accord also with general experience is the high incidence of men with duodenal ulcer, the ratio of men to women being 4.8 to 1. The small number of women in this study, however, made it impossible to determine statistically whether or not their plasma pepsinogen values were significantly lower than those of similar aged men as is alleged to occur with respect to gastric secretion. It is of interest to note that the ratio of men to women in the 14 per cent of the control group with plasma pepsinogen values beyond the mean of the group with duodenal ulcer was 2.8 to 1 as contrasted with a ratio of 1.07 to 1 for the whole control group.

Many of the subjects comprising the group with duodenal ulcer did not present any signs or symptoms of an active ulcer at the time of the present study. Although a tendency for higher values was noted among patients with active lesions, there was no statistically significant difference between those with and without active lesions. Likewise, no significant correlation could be established between the values for plasma pepsinogen and the interim between the time of study and that when the active lesion was apparent.

It would appear from the preceding that the plasma pepsinogen of a patient with peptic ulcer may remain augmented even after the lesion has healed and the patient is free of symptoms. In accord are the observations on pepsinogen excretion in the urine of subjects with duodenal ulcer whose high excretory rates remained unchanged long after the lesion had healed.[16] Likewise, Levin, Kirsner, and Palmer[17] have observed that the nocturnal secretion by the stomach of the patient with duodenal ulcer may show no significant change even after the ulcer has healed.

If it be true that hypersecretion by the stomach, as reflected in the plasma pepsinogen, may continue even after the duodenal ulcer has healed, then it becomes pertinent to inquire as to whether such hypersecretion may have occurred long before the development of the lesion. During the course of studies on the excretion of pepsinogen in the urine of apparently healthy subjects, an occasional subject has been encountered whose excretory rate was well within the range of those of patients with active duodenal ulcers. One such subject, whose urines were assayed almost daily for about a year, developed the typical signs and symptoms of a duodenal ulcer which was subsequently demonstrated roentgenologically. Yet no appreciable change in pepsinogen excretion was noted. Another subject with a high rate of pepsinogen excretion was studied similarly for almost two years when he developed an acute perforation as the first sign of the presence of a duodenal ulcer. Both subjects had high plasma pepsinogen values, but, because of the absence of any signs or symptoms of a gastrointestinal disturbance, they were classified as

"healthy" and regarded as controls. These two subjects were in the 14 per cent of apparently healthy subjects with values beyond the mean of the duodenal ulcer group.

Available evidence does not permit any inferences with respect to the future course of the individual whose plasma pepsinogen content is within the range of the patient with duodenal ulcer, and presumably has a stomach which is hypersecreting with respect to pepsinogen. It may well be that such an individual *can* develop a duodenal lesion but at the present time it is impossible to predict that he *will* develop such a lesion. Only the study of such individuals over a long period of time will yield the answer as to whether the subject with a high plasma pepsinogen is more susceptible to ulcer than is the subject with a lower level. Studies designed for such purpose are now in progress.

The plasma pepsinogen values observed in patients with pernicious anemia differ significantly from those of subjects without this syndrome (Fig. 6). In no instance was any value obtained in a patient with pernicious anemia which was above the mean of the subjects comprising the control group, while none of the control group had values below the mean of the group with pernicious anemia. Approximately 2 per cent of the subjects in the control group had values within one standard deviation of the mean for all subjects with pernicious anemia.

The observation that there is some pepsinogen in the plasma of the patient with pernicious anemia is in accord with the studies of Helmer, Fouts, and Zerfas[18] who found that the peptic activity of gastric juice from patients with pernicious anemia was about $\frac{1}{100}$ of that of healthy persons. Such low values and the highly significant differences from the control group suggest that the determination of the plasma pepsinogen may serve as a valuable aid in the diagnosis of this syndrome.

Just as with hypersecretion, it is pertinent to inquire into the significance of gastric hyposecretion as reflected by the plasma pepsinogen. Toward that end, complete hematologic studies were made on a number of subjects whose plasma pepsinogen values were within the range of those of patients with pernicious anemia. In no instance were any abnormalities observed at the time. Yet, within six months, one subject, aged 68 years, developed all the signs and symptoms of the blood dyscrasia, and subsequently the diagnosis was established by typical morphological changes in the blood and bone marrow.

The preceding suggest that the plasma pepsinogen may be a valuable aid in the diagnosis of duodenal ulcer and pernicious anemia. The significance of high or low values in apparently healthy subjects, however, must await the accumulation of extensive data on many persons.

CONCLUSIONS

The pepsinogen content of the plasma is fairly constant from hour to hour and day to day. The ingestion of food does not affect the level characteristic for any individual.

The pepsinogen content of the plasma is significantly higher in patients with duodenal ulcer than in apparently healthy subjects of the same age.

The plasma of patients with pernicious anemia contains significantly less pepsinogen than that of apparently healthy subjects of the same age.

In spite of overlapping of the plasma pepsinogen values of the control and duodenal ulcer group, the differences are such as to suggest that the assay of plasma pepsinogen may serve as a valuable aid in the diagnosis of duodenal ulcer. Likewise, the assay of plasma pepsinogen may prove valuable as an aid in the diagnosis of pernicious anemia.

The significance of very high and very low plasma pepsinogen values among the apparently healthy population is discussed. The role of such values as indices of susceptibility to duodenal ulcer and pernicious anemia is indicated.

The technical assistance of Miss Doris Riedinger is gratefully acknowledged.

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SPECIATION WITHIN THE GENUS BRUCELLA

I. Dye Sensitivity of Smooth Brucellae

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SPECIATION in the genus *Brucella* is usually established by reference to relative rate of growth, carbon dioxide requirement, hydrolysis of urea, reduction of nitrate, production of sulfide, dye sensitivity, and specific agglutination in absorbed antisera. No one, or combination, of these criteria is completely satisfactory for identification of field strains, however, because of the close similarity of the species. For example, most, but not all, strains of *Br. suis* grow more rapidly than do those of the other two species; some strains of *Br. abortus* do not require added carbon dioxide for growth; and a distinction between *Br. abortus* and *Br. suis* cannot be made serologically. These and other limitations have been discussed by several investigators.[1-3] Nevertheless, and despite its recognized limitations, the dye sensitivity test remains one of the most practical aids for differentiating the species. It formerly had the disadvantage that stock solutions of the dyes were relatively unstable and that, as ordinarily employed, several plates were required for the complete test. The test proposed by Cruickshank[4] largely obviates these difficulties but introduces some irregularities in the amount of dye present in different lots of impregnated paper strips. The test described below which employs dye-tablets rather than paper strips in an adaptation of the Cruickshank procedure offers the combined advantages of simplicity and reproducibility.

MATERIALS AND METHODS

Media.—Albimi's brucella broth, enriched with 0.0001 per cent each of nicotinamide and thiamin and solidified with 2 per cent agar, was employed for most of the experimental work. For some of the later work, however, this medium was additionally enriched with 0.0004 per cent hemin.[5]

Test Strains of Brucellae.—Twenty-one of the thirty test cultures were received from, and had been speciated by, reference laboratories. Two were recovered from human blood cultures by local laboratories and were speciated by us. Five were recovered from human blood cultures by us; and two were derived, by animal passage, from mucoid brucella variants. All cultures were plated and examined for purity before use, and all appeared to be colonially and microscopically smooth. The dye sensitivity tests were made with heavy (approx. 10^{10} organisms per ml.) suspensions obtained from 48- to 72-hour slant cultures. Each experimental plate was streaked with four parallel, straight-line inocula from a single suspension, using one 4-mm. loopful for each line. Two illustrative plates are shown in Fig. 1.*

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*We are grateful to Zane Price, medical photographer, for preparation of the photographs used in this figure.

Dyes.—These were National Aniline products, and the concentrations specified below are *not* corrected for impurities (the claimed dye contents were: thionin, 79 per cent; basic fuchsin, 96 per cent; crystal violet, 94 per cent; and pyronin Y, 25 per cent).

For the Cruickshank paper strip procedure, solutions of the dyes in 50 per cent ethanol were first prepared at the recommended concentrations.[4] These concentrations were later changed, on the basis of exploratory experiments, to 1:800 (weight:volume) thionin, 1:200 fuchsin, 1:200 crystal violet, and 1:1,600 pyronin. Each set of papers was prepared by placing fifty 5 by 70 mm., strips (Whatman No. 1 filter paper) in a 20 by 150 mm. Pyrex tube, adding 2.5 ml. of dye solution, setting aside for twenty-four hours to permit uniform distribution of the dye, steam sterilizing, and drying at 120° C.

Tablets were prepared* such that the final concentrations were near those obtaining in the solutions above, namely, 1:800 (weight:weight), 1:200, 1:400, and 1:800, respectively. These were found not to require sterilization before use.

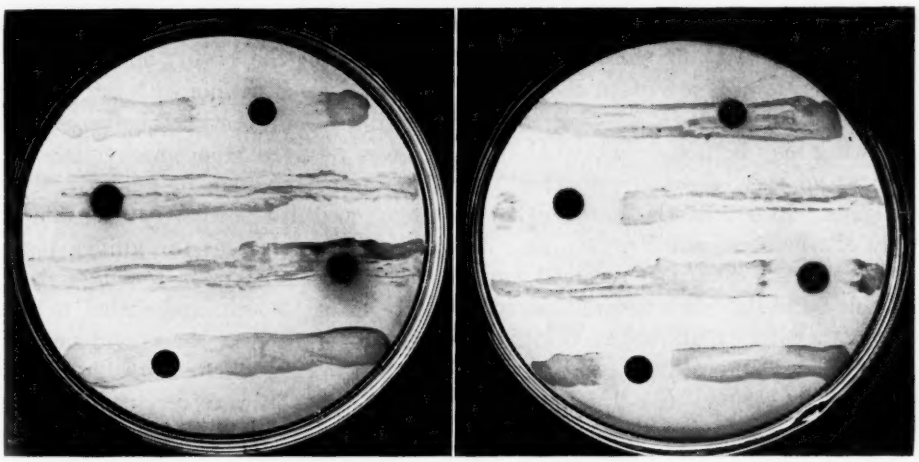

Fig. 1.—Two dye-tablet differential plates. Left, *Br. abortus;* right, *Br. suis.* Top to bottom: thionin, fuchsin, crystal violet, pyronin.

Finally, as a modification of the paper strip procedure, paper discs were also tested. These required slightly different concentrations of the dyes, namely, 1:200 thionin, 1:100 fuchsin, 1:100 crystal violet, and 1:2,000 pyronin. Discs of 8 mm. diameter were cut from chromatographic paper (Schleicher and Schuell No. 470-A) which had been saturated with solutions of the dyes and dried. The discs were steam sterilized and dried at 120° C.

Incubation and Reading.—In most instances, and for best results, prepared plates were held fourteen to sixteen hours at 4° C. to permit some diffusion of dyes into the medium prior to growth of the organisms. The plates were then incubated forty-eight hours at 35° C. under 10 per cent carbon dioxide. Dye sensitivity was recorded in terms of millimeter inhibition (from margin of growth to edge of paper or tablet).

RESULTS

Results obtained from tests by the three methods are presented in Table I. Some technical difficulties were encountered in obtaining satisfactory and reproducible plates in the paper strip tests. The procedure finally followed, consisted of layering 10 ml. of medium in a plate, carefully depositing, in parallel, two paper strips on this "cementing layer" of medium, and then layering a

*Compounded by Boyle & Co., Los Angeles, the binder being a standard granulation consisting of 88 parts dicalcium phosphate, 10 parts starch, 2 parts acacia, and a trace of calcium stearate.

second 10 ml. of medium over the strips. Upon adoption of this procedure, results indicative of the species[4, 6] were obtained with three strains of *Br. melitensis* and seven strains of *Br. suis*. No concentrations of the dyes were found, however, which also gave satisfactory results with the seven strains of *Br. abortus* which were under test at this time.

The dye-discs were, technically, more convenient than dye-strips, but retained the disadvantage that results were not readily reproducible, and the dye content appeared to vary in different preparations of the discs.

Tablets have been shown to be convenient vehicles for antibiotic sensitivity tests,[7, 8] and we have found them to be equally well suited for testing dye sensitivity. The results obtained from tests with dye tablets are expressed qualitatively in Table II, in which a "dye sensitivity" of less than 4 mm. is recorded as "–", and 4 mm. or more as "+". Accordingly, of the twenty-one pretyped cultures examined, seventeen showed dye sensitivities compatible with the species to which each had been assigned. Of the remaining four pretyped cultures, the following may be noted: (1) "Abortus 3c" was received from another laboratory as an "avirulent *Br. abortus*" suitable for preparation of agglutinating antigens. In our hands, however, it gave urease and dye sensitivity tests which were characteristic of *Br. suis*, and it was distinctly virulent for guinea pigs. (2) "Abortus 3f", also received from another laboratory, was for us a nontypable brucella. It appeared microscopically to be smooth, and failed to agglutinate with acriflavine.[9] Colonially, however, it showed some evidence of dissociation to mucoid and, serologically, it failed to agglutinate with high titer antibrucella serum. (3) "Melitensis 4a" and abortus 3a were received in 1945 and both had been retained as lyophylized cultures since 1947. When examined during the course of these studies, the two gave identical sulfide production, nitrate reduction, CO_2-independence, urease, and dye sensitivity tests; and both were agglutinated by absorbed antiabortus but not by absorbed antimelitensis serum. It appeared, therefore, that "melitensis 4a" was in reality a copy of abortus 3a. (4) Finally, "melitensis 4h," recently received from another laboratory, behaved, rather, like a relatively dye-fast *Br. suis* (the zones of inhibition were: thionin, 0; fuchsin, 0; crystal violet, 6; and pyronin, 5 mm.). Its urease and sulfide reactions and agglutination with absorbed antiabortus but not with absorbed antimelitensis serum permitted no alternative conclusion.

TABLE I. GROWTH INHIBITION OF SELECTED BRUCELLA STRAINS IN THE PRESENCE OF DYE-STRIPS, DYE-DISCS, AND DYE-TABLETS

TEST STRAIN	STRIP				DISC				TABLET			
	MM. INHIBITION BY*:											
	T	F	V	P	T	F	V	P	T	F	V	P
abortus 3a	22	5	0	0	18	0	0	4	21	2	3	3
abortus 3i	0	0	0	0	14	0	0	3	11	1	1	0
abortus 3h	0	0	0	0	11	0	0	8	12	0	0	0
melitensis 4d	0	0	0	0	4	0	0	4	2	0	0	0
melitensis 4c	0	0	0	0	0	0	0	0	2	1	1	0
suis 3s-3	0	23	16	24	0	8	9	20	0	12	12	12
suis 3s-4	0	25	14	22	2	7	8	16	1	10	13	9

*T, thionin; F, basic fuchsin; V, crystal violet; P, pyronin Y.

Of the seven strains of brucellae speciated by us, and which had been recovered from human blood cultures, five were readily typed by the dye-tablet procedure. Number V-1 was a typical CO_2-dependent *Br. abortus;* No. 128 was CO_2-independent but gave the biochemical and serological (absorbed sera) reactions of *Br. abortus;* No. 141 was slightly pyronin sensitive (7 mm. zone of inhibition) but, like No. 206, was biochemically and serologically a typical *Br. melitensis;* and No. V-2 was a typical *Br. suis* according to biochemical, as well as dye sensitivity, tests.

Strains No. 21a and 21c were recovered from siblings in a family of five, all of whom had symptoms of chronic brucellosis. These two strains, like abortus 3f, were slightly mucoid serologically (i.e., stringy, rather than granular, agglutination). Neither agglutinated with low titer absorbed antisera nor with normal serum, but both agglutinated to partial titer with a high titer anti-brucella serum. No. 21c, but not 21a, gave a positive acriflavine test.[9] Neither, like abortus 3f, gave a recognizable pattern in dye sensitivity tests though both, like several mucoid brucella variants which we have examined, were rather sensitive to fuchsin and crystal violet, but relatively insensitive to thionin and pyronin.

Finally, the two derived strains of brucellae were readily typed as *Br. suis* by the dye-tablet procedure, and these results agreed with those obtained from

TABLE II. SENSITIVITY TESTS ON STOCK AND FIELD STRAINS OF BRUCELLAE, DYE-TABLET PROCEDURE

TEST STRAIN	SENSITIVE TO: T	F	V	P	REMARKS
abortus 3a	+	−	−	−	typical abortus
abortus 3c	−	+	+	+	typical suis*
abortus 3d	+	−	−	−	typical abortus
abortus 3f	+	+	+	+	nontypable*
abortus 3h	+	−	−	−	typical abortus
abortus 3i	+	−	−	−	typical abortus
abortus 3j	+	−	−	−	typical abortus
abortus 3k	+	−	−	−	typical abortus
abortus 3m	+	−	−	−	typical abortus
melitensis 4a	+	−	−	−	typical abortus*
melitensis 4b	−	−	−	−	typical melitensis
melitensis 4c	−	−	−	−	typical melitensis
melitensis 4d	−	−	−	−	typical melitensis
melitensis 4h	−	−	+	+	fuchsin-fast suis*
suis 3s-1	−	+	+	+	typical suis
suis 3s-2	−	+	+	+	typical suis
suis 3s-3	−	+	+	+	typical suis
suis 3s-4	−	+	+	+	typical suis
suis 3s-5	−	+	+	+	typical suis
suis 3s-8	−	+	+	+	typical suis
suis 3s-9	−	+	+	+	typical suis
blood culture V-1	+	−	−	−	typical abortus
blood culture V-2	−	+	+	+	typical suis
blood culture 21a	−	+	+	−	nontypable*
blood culture 21c	−	+	+	−	nontypable*
blood culture 128	+	−	−	−	typical abortus
blood culture 141	−	−	−	+	pyronin-sensitive melitensis*
blood culture 206	−	−	−	−	typical melitensis
from mucoid 3cSB	−	+	+	+	typical suis
from mucoid 33M	−	+	+	+	typical suis

*See comment in text.

biochemical tests. One of these strains, No. 3c, was derived by animal passage from a mucoid variant which had been maintained for nearly two years in our culture collection.[10] This mucoid variant, in turn, was originally derived in vitro from "abortus 3c" (see above). The other strain, No. 33, was originally recovered as a mucoid brucella variant from a patient who had suffered intermittently for thirty years with chronic brucellosis, following an initial attack of acute brucellosis. This mucoid strain had been maintained in our collection approximately one year prior to its animal passage and conversion to a smooth brucella.

DISCUSSION

Use of dicalcium phosphate tablets containing the dyes which characterize species in the genus *Brucella* is a convenient and quite satisfactory method for typing these organisms. Our data indicated, in agreement with those of Cruickshank,[4] that all of the dyes—not just thionin and fuchsin—should be included in the dye sensitivity test. Irregularities in the sensitivity patterns occurred in only two instances among the 27 completely smooth brucellae which were examined by this procedure. With these two strains, satisfactory speciation was obtained by reference to serological and biochemical tests. Several points may be noted regarding the latter, though we, of course, did not examine a sufficient number of strains to permit the drawing of definite conclusions. Of the several biochemical tests employed, hydrolysis of urea was found to be particularly helpful. Under our experimental conditions (essentially the medium of Rustigian and Stuart,[11] tubed in one ml. amounts, and inoculated with approximately 10 million bacteria), all of twelve strains of *Br. suis* gave positive tests within 15 minutes, whereas no strain of *Br. abortus* or *Br. melitensis* gave a positive test in less than 55 minutes. These results are in complete agreement with those of Pacheco and Thiago de Mello,[12] who also found that strains of *Br. suis* gave much more rapid urease tests than did those of the other two species. Further, the urease test appears also to serve as a rather sensitive indicator for nonsmooth brucella variants in that these give either delayed or negative, tests.

In our hands nitrate reduction also served as a useful guide for speciation, since all five strains of *Br. melitensis* gave either negative or very weakly positive tests for nitrite, while all of twenty-two strains of *Br. abortus* and *Br. suis* gave either moderately or strongly positive tests. The latter species differed, however, in that aged (ten days' incubation) nitrate broth cultures of *Br. suis* frequently gave negative tests for nitrite. It is interesting to note that these results, and even more clearly those of Zobell and Meyer,[13] suggest the hypothesis that, as a readily available source of oxygen, *Br. suis* is capable of using both nitrates and nitrites, *Br. abortus*, only nitrates, and *Br. melitensis*, neither.

Finally, in respect to biochemical tests, we, like Brim and associates,[14] failed to find sulfide production of appreciable help toward speciating these organisms. Several strains of *Br. abortus*, and one strain of *Br. melitensis*, like all strains of *Br. suis* examined, gave positive sulfide tests through five days of incubation. In a somewhat similar sense, CO_2-independence had but limited significance, since only four of our ten strains of *Br. abortus* were CO_2-dependent.

SUMMARY

A differential dye sensitivity test is described which permitted satisfactory speciation of twenty-five strains of smooth brucellae. Two slightly irregular strains were speciated by reference to their biochemical and serological reactions. Three strains could not be typed, but all were found to be nonsmooth.

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MODIFIED GLOBIN

III. Determination of Modified Human Globin in Blood Serum and in Urine

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OTHER publications[1-5] from this laboratory have given information on the preparation, the nature of the modification, and some of the clinical uses of modified globin prepared from human erythrocytes. This paper presents methods for the determination of modified human globin in blood serum and in urine.

Originally the modified human globin concentration in blood serum was determined turbidimetrically after precipitation with commercial Bacto Tryptone and in urine after precipitation with sulfosalicylic acid. These procedures, although they were not extensively investigated, were discontinued, and a more promising method devised by Kendall and Abell[6] was adopted. In this method, modified globin is selectively precipitated by trichloracetic acid buffered at pH 4.7 with sodium citrate, and the quantity of nitrogen in the precipitate, as determined by the Kjeldahl method, serves as a measure of modified globin.

We found the Kendall-Abell method quite reliable; however, in order to simplify and shorten it, and to eliminate the possibility of losing precipitate in the necessary transfers from one container to another, we modified the method so that it is possible to carry out the entire procedure in a colorimeter tube, without transfers, and to determine the amount of precipitated modified globin by measuring the turbidity in a photoelectric colorimeter.

Since these methods have not appeared in the generally available literature, they will be presented in detail here.

METHOD

Principle.—A solution of trichloracetic acid buffered at pH 4.7 with sodium citrate selectively precipitates modified globin from diluted blood serum or urine, and the precipitate, which is proportional to the amount of modified globin present, may be measured turbidimetrically or by analyzing it for nitrogen by the Kjeldahl or other suitable method.

Equipment.—Klett-Summerson or other suitable photoelectric colorimeter, with standardized test tubes and a filter that has a maximum transmission at 420 millimicrons.

Reagents.—Kendall-Abell Reagent: Dissolve 81.7 Gm. of trichloracetic acid and 147 Gm. of tribasic sodium citrate dihydrate in distilled water and dilute to volume in a one-liter volumetric flask.

From the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation, Bryn Mawr Hospital.

This paper is the twenty-fifth of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation of the Bryn Mawr Hospital. The work described here was aided by a grant from the John S. Sharpe Research Foundation and was in part carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Bryn Mawr Hospital and a contract with the Army Medical Research and Development Board.

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Sodium chloride, 0.9 per cent w/v
Sodium hydroxide, 0.1000N
Hydrochloric acid, 0.1000N
Trichloracetic acid, 10 per cent w/v

Gum ghatti: Tie 3 Gm. of gum ghatti tears in a double gauze bag and place in 100 ml. of water to stand overnight. After the standing period, remove the gauze bag, squeeze it gently, and discard. If the solution contains solid material, filter it through a plug of cotton. The solution should be refiltered if a precipitate appears on standing.

Stock globin standard: A bottle from a production lot which contains a 4 per cent (approximately) sterile solution of modified human globin. This solution must be analyzed for nitrogen by the Kjeldahl method to determine the exact amount of globin present. When corrected for moisture, ash, anion, and cation, the nitrogen content of modified human globin is 16.68 per cent. A *sterile* standard solution of modified globin is stable for at least two years at room temperature.

Dilute globin standard, 1 mg. per milliliter: Dilute the stock globin standard with 0.9 per cent w/v sodium chloride to make a solution containing exactly 1 mg. of modified globin per milliliter. This solution should be stable for about one week if stored at 4° C.

Dilute globin standard, 5 mg. per milliliter: Dilute the stock globin standard with 0.9 per cent w/v sodium chloride to make a solution containing exactly 5 mg. of modified globin per milliliter. This solution should be stable for about a week if stored at 4° C.

Procedure.—Nitrogen method for blood serum: Into individual 15 by 125 mm. test tubes for each sample introduce the following materials in the order given, left to right, mixing by lateral shaking after each addition:

TABLE I

	SERUM* (ML.)	5 MG./ML. DILUTE STANDARD (ML.)	0.9% W/V NaCl (ML.)	REAGENT (ML.)
Standard 1	0.5	1	1	2.5
Standard 2	0.5	2		2.5
Unknowns	0.5	1	1	2.5

*The serum for both standards should be collected from the patient whose blood is to be analyzed for globin before globin is infused or 24 hours after infusion.

Let tubes stand at room temperature for one hour.

Centrifuge tubes for 1.5 hours at 4,000 r.p.m.

Carefully decant and discard the supernatant solution so that none of the sediment is lost.

Add 1 ml. of 0.1N sodium hydroxide to each tube and shake to dissolve the sediment.

Reprecipitate the globin by adding 1 ml. of 0.1N hydrochloric acid and 2 ml. of the Kendall-Abell reagent.

Centrifuge the tubes for thirty minutes at 4,000 r.p.m.

Carefully decant and discard the supernatant solution so that none of the sediment is lost.

Dissolve the sediment in 5 ml. of 0.1N sodium hydroxide, and analyze it for nitrogen by the Kjeldahl or other suitable method.

The amount of globin in unknowns, after subtracting the amount added, is proportional to the amount in the standards.

Nitrogen method for urine: Adjust the pH of urine specimens to 4.7 with the aid of a pH meter or bromcresol green indicator, and filter through Whatman No. 1 paper.

In a 15 by 125 mm. test tube mix 5 ml. of the filtered urine with 5 ml. of the Kendall-Abell reagent, and let the tube stand at room temperature for thirty minutes.

Centrifuge the tubes for one hour at 4,000 r.p.m.

Carefully decant and discard the supernatant solution so that none of the sediment is lost.

Dissolve the sediment in 4 ml. of 0.1N sodium hydroxide, and add 4 ml. of 10 per cent trichloracetic acid to reprecipitate the globin.

Centrifuge the tube for thirty minutes at 4,000 r.p.m.

Carefully decant and discard the supernatant solution so that none of the sediment is lost.

Dissolve the sediment in 5 ml. of 0.1N sodium hydroxide, and analyze it for nitrogen by the Kjeldahl or other suitable method.

Calculation:

mg. nitrogen × 7.5 = mg. globin/5 ml. urine.

Turbidimetric method for blood serum: Into individual Klett tubes for each sample introduce the following materials in the order given, left to right, and mix by lateral shaking after each addition:

TABLE II

	SERUM* (ML.)	0.9% W/V NaCl (ML.)	1 MG./ML. DILUTE STANDARD (ML.)	REAGENT (ML.)
Blank	0.5	4.5		5
Standard 1	0.5	3.5	1	5
Standard 2	0.5	2.5	2	5
Standard 3	0.5	1.5	3	5
Standard 4	0.5	0.5	4	5
Unknowns	0.5	4.5		5

*Serum for blanks and standard should be collected from the patient whose blood is to be analyzed for globin before globin is infused or twenty-four or more hours after infusion.

Mix tubes twice by inversion after addition of the reagent.

Allow tubes to stand for two hours at room temperature.

Mixing each tube twice by inversion immediately before reading, read in the colorimeter which has been set to zero with distilled water, using a filter that has a maximum transmission at 420 millimicrons.

Subtract the blank reading from standard readings, and plot the resulting figures against standard concentrations on linear graph paper. A straight line which does not pass through zero should be obtained.

Unknown readings, after the blank reading has been subtracted, are used in reading globin concentrations from the standard curve.

Turbidimetric method for urine: Into individual Klett tubes for each sample introduce the following materials in the order given, left to right, and mix by lateral shaking after each addition:

TABLE III

	URINE* (ML.)	0.9% W/V NaCl (ML.)	1 MG./ML. DILUTE STANDARD (ML.)	GUM GHATTI (ML.)	KENDALL REAGENT (ML.)
Blank 1	2	9		2	
Standard 1	2	3	1	2	5
Sandard 2	2	2	2	2	5
Standard 3	2	1	3	2	5
Blank 2	2	9		2	
Unknowns	2	4		2	5

*Urine for blank 1 and standards should be collected from each patient before administration of globin, when post-infusion urine is to be analyzed for modified globin. Use unknown urine for blank 2.

Mix twice by inversion after addition of the reagent.

Allow to stand for two hours at room temperature.

Mixing each tube twice by inversion immediately before reading, read in the colorimeter, set to zero with distilled water and using a filter that has a maximum transmission at 420 millimicrons.

Subtract the blank 1 reading from standard readings, and plot the resulting figures against standard concentrations on linear graph paper. A straight line, which does not pass through zero, should be obtained.

Unknown readings, after the blank 2 reading has been subtracted, are used in reading globin concentrations from the standard curve.

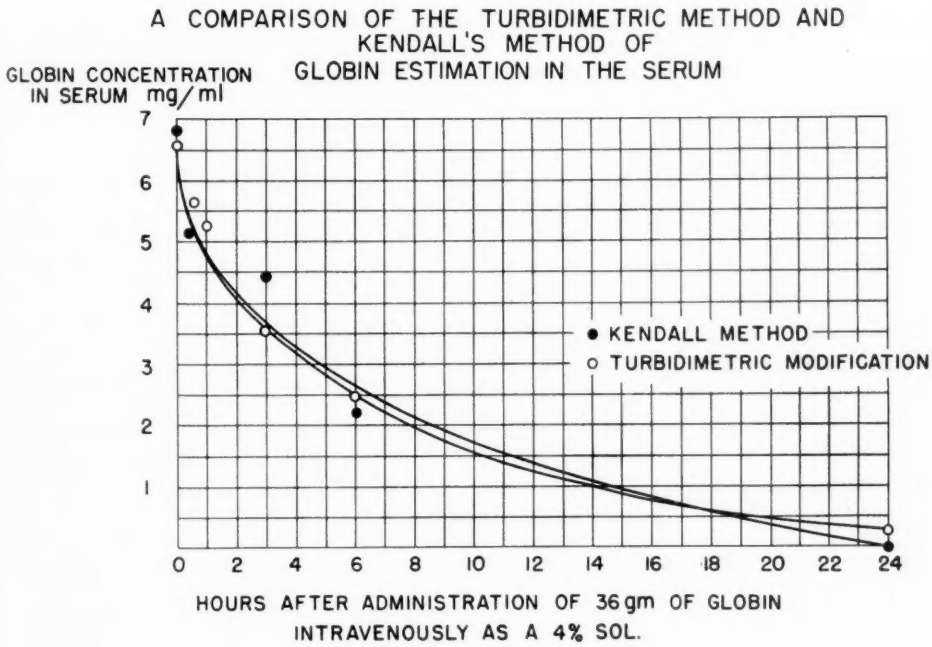
A COMPARISON OF THE TURBIDIMETRIC METHOD AND KENDALL'S METHOD OF GLOBIN ESTIMATION IN THE SERUM
GLOBIN CONCENTRATION IN SERUM mg/ml
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24
● KENDALL METHOD
○ TURBIDIMETRIC MODIFICATION
HOURS AFTER ADMINISTRATION OF 36 gm OF GLOBIN INTRAVENOUSLY AS A 4% SOL.

Fig. 1.

DISCUSSION

It appears that modified globin disappears from the blood serum within twenty-four hours after the last injection, so serum collected before or twenty-four hours after the last infusion of modified globin is suitable for use in blanks and standards.

The nitrogen method for determining modified globin in blood serum is not sufficiently sensitive to analyze serum containing less than one per cent w/v of modified globin, so this explains the addition of a known amount of modified globin to unknowns.

The factor 7.5 was obtained by analyzing a number of different urines containing known amounts of modified globin. This factor should be determined for each new lot of modified globin.

It was found unnecessary to add the Kendall-Abell reagent to urine blanks, since blank readings are largely dependent on the amount of color in the urine. This also explains why separate blanks are necessary for unknowns and standards. Any globin-free urine may be used for blank 1 and standards.

In the modified method, it was not found necessary to adjust the pH of urine to 4.7.

It is advisable to do the determinations (blanks, standards, and unknowns) in duplicate and to take the averages of data obtained.

Fig. 1 shows the correlation of results obtained with the N method (Kendall-Abell) and the turbidimetric method of estimation of globin in serum.

SUMMARY

Methods for the determination of modified human globin in blood serum and in urine are presented.

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MODIFIED GLOBIN

IV. Some of the Physiological Properties of Modified Human Globin

Max M. Strumia, M.D., John J. McGraw, Jr., M.D., and
Albert B. Sample, M.S., Bryn Mawr, Pa.

INTRODUCTION

PREVIOUS papers from this laboratory have outlined the preparation of modified globin,[1] the method of determination in the blood and urine,[2] and some of the clinical applications.[3-5]

The present paper deals with some of the physiological properties of modified globin. Particular emphasis is placed upon its effect on plasma volume and plasma viscosity, the retention of globin in the blood stream, and its excretion in the urine.

MATERIALS AND METHODS

The bulk of the modified globin used in this study was prepared and furnished by Sharp and Dohme, Inc., of West Point, Pa. Some of the material used in earlier experiments was prepared by Smith, Kline & French Laboratories of Philadelphia, Pa. Most of the globin was used as a 4 per cent concentration in a 5 per cent solution of glucose. Globin solutions for intravenous use must be made isotonic with glucose or sodium chloride.*

Plasma volumes were determined by the method of Gibson and Evelyn[6] using T-1824 (Evans Blue dye). In some instances when there was no reason to believe that there were any variations in the red cell mass, changes in plasma volume, for periods up to twenty-four hours subsequent to the original measurements, were determined by calculation of the hematocrit using the high speed centrifuge-capillary tube method[7] with a correction of −6.8 per cent.

The variations of the plasma volume based on the original determination of the plasma volume by the dye method and repeated determinations of the hematocrit before and after injections of various solutions were obtained with the use of the formula:

$$PV = \frac{dv \times het}{nhet}$$

where PV is the unknown plasma volume; dv is the original plasma volume determined by the dye method; het is the corrected hematocrit obtained at the time of the original plasma volume determination and nhet is the new hematocrit value.

This work was carried out in the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation of the Bryn Mawr Hospital over a period of years with the help and cooperation of Dr. Joseph Harrison, Dr. Alton D. Blake, Jr., Dr. Maurice R. Nance, Dr. Charles Catanzaro, and Dr. Vittorio Satta, with the technical aid of Margaret Dolan, M.T., and Louise S. Colwell, M.T.

This paper is the twenty-sixth of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation of the Bryn Mawr Hospital. The work described here was aided by a grant from the John S. Sharpe Research Foundation and was, in part, carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Bryn Mawr Hospital.

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*Most units of modified globin, as prepared at present by Sharp and Dohme Laboratories, contain approximately 7.0 Gm. of NaCl for each 100 Gm. of dry substance. A 4 per cent isotonic solution of this globin therefore could, strictly speaking, be obtained by dissolving the content of a standard unit (containing 16 Gm. of globin + 1.2 Gm. of NaCl) in 400 ml. of 3.2 per cent glucose or 400 ml. of 0.6 per cent NaCl. Practically, however, in most clinical conditions, either 5 per cent dextrose or 0.85 per cent NaCl are used to dissolve globin.

It is recognized that this method is subject to considerable variations; therefore the values were generally checked at proper intervals by determinations of plasma volume by the dye method.

The oncotic value of globin was estimated by dividing the measured increase in plasma volume by the grams of globin injected.

Modified Ostwald viscosity tubes, described by Cannon and Fenske,[8] were used to measure the plasma viscosity.

RESULTS

Variations in Plasma Volume Following Injections of Globin.—All of the plasma volume experiments described below were conducted in a similar manner, except for variations in the general physical condition of some of the recipients and the volumes of material injected.

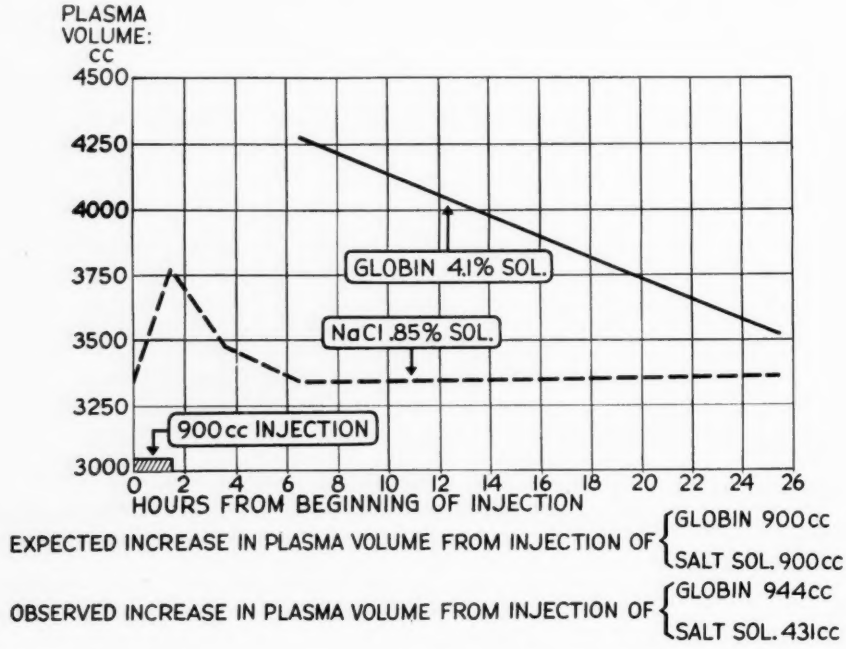
PLASMA VOLUME: CC
4500
4250
4000
3750
3500
3250
3000
GLOBIN 4.1% SOL.
NaCl .85% SOL.
900cc INJECTION
0 2 4 6 8 10 12 14 16 18 20 22 24 26
HOURS FROM BEGINNING OF INJECTION
EXPECTED INCREASE IN PLASMA VOLUME FROM INJECTION OF {GLOBIN 900cc / SALT SOL. 900cc
OBSERVED INCREASE IN PLASMA VOLUME FROM INJECTION OF {GLOBIN 944cc / SALT SOL. 431cc

Fig. 1.—W. Y., man, aged 64 years. H. 185 cm.; W. 75 kg.; expected normal plasma volume 3,498 c.c.

All of the results could have been expressed in terms of plasma volume increase, but in order to illustrate different aspects of the problem with maximum clarity the results of some experiments have been charted as percentage increase in the recipient's plasma volume, oncotic value of globin, etc.

One experiment illustrates the effect on the plasma volume of equal quantities of 0.85 per cent solution of sodium chloride compared with 4.1 per cent globin. Two convalescing patients were tested. The results may be seen in Figs. 1 and 2. Briefly, the infusion of 900 ml. of salt solution in one patient resulted in an elevation of the plasma volume of 431 ml. at the end of the ninety-minute period required for the injection. The globin solution showed

complete retention for six hours with a gradual return to normal. In the second patient the plasma volume was not measured until four hours after the injection of salt solution. The elevation of plasma volume at that time was negligible, but this patient showed maximum rise four hours after globin transfusion, returning to normal in twenty-four hours.

The two cases illustrated in Figs. 1 and 2 demonstrate maximal effects from infusions of globin. Such results are not always obtained, but a significant increase of plasma volume may be expected consistently. This is illustrated by studies done on a series of eight convalescing patients, all of whom were hypoproteinemic. They were given 4 per cent globin in sufficient volume to produce, if entirely retained, a 25 per cent increase in their plasma volume.

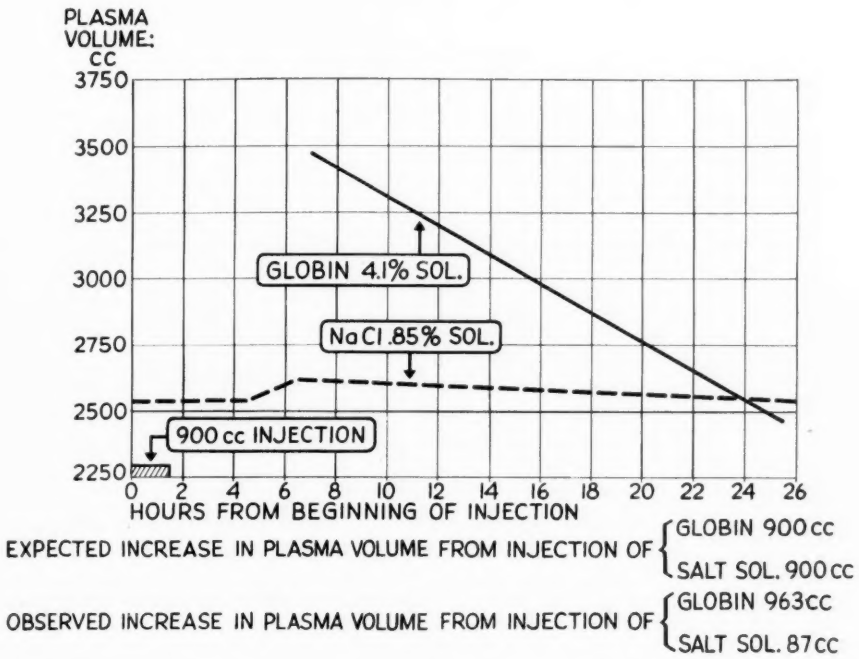
PLASMA VOLUME: CC
3750
3500
3250
3000
2750
2500
2250
GLOBIN 4.1% SOL.
NaCl .85% SOL.
900 cc INJECTION
0 2 4 6 8 10 12 14 16 18 20 22 24 26
HOURS FROM BEGINNING OF INJECTION
EXPECTED INCREASE IN PLASMA VOLUME FROM INJECTION OF { GLOBIN 900 cc / SALT SOL. 900 cc
OBSERVED INCREASE IN PLASMA VOLUME FROM INJECTION OF { GLOBIN 963 cc / SALT SOL. 87 cc

Fig. 2.—A. S., man, aged 66 years. H. 152 cm.; W. 45 kg.; expected normal plasma volume 2,805 c.c.

The results are seen in Fig. 3. Three of the plasma volumes increased from 11 to 14 per cent, two increased 18 per cent, and three, 27 per cent. Thus, the plasma volume increases varied between 50 and 100 per cent of the maximal expected.

Expressed in another way, the experiments described above are a measure of the oncotic value of globin. They show that 4 Gm. of globin in 100 ml. of solution produce increases of plasma volume of approximately 50 to 100 ml. Thus, each gram of globin must hold from 12.5 to 25 ml. of water in the vascular system, under the experimental conditions.

In order to compare globin with plasma, another group of experiments was carried out with the cooperation of a single recipient, whose hemodynamics were essentially normal. After the plasma volume had been measured he received four infusions of 400 to 1,200 ml. of 4 per cent globin on four different days, with an interval of at least one day. Variations in his plasma volume were calculated from hematocrit studies at 0, 1, 2, 3, 4, and 5 hours. On two

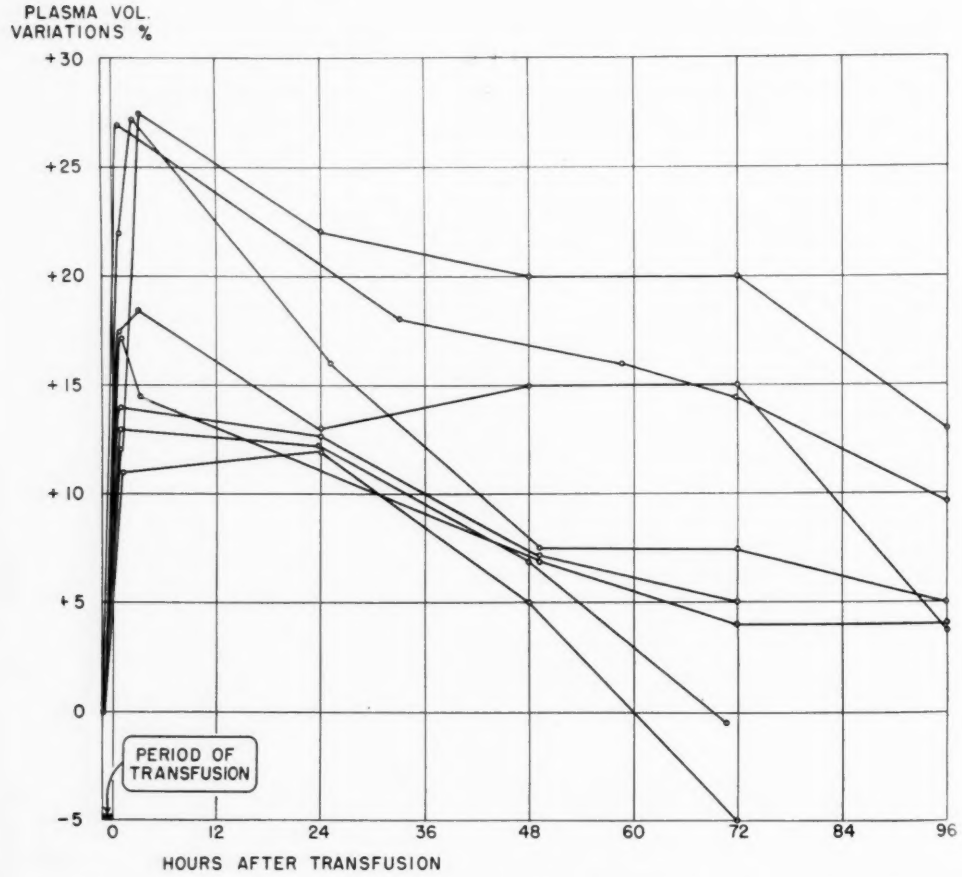

Fig. 3.—Effect of intravenous injection of globin on plasma volume hypoproteinemic patients.

TABLE I. INCREASE IN PLASMA VOLUME IN A SINGLE RECIPIENT WITH ESSENTIALLY NORMAL HEMODYNAMICS

	ML. INCREASE OF PLASMA VOLUME AFTER INFUSION		
	0 HOURS	2 HOURS	4-5 HOURS
400 ml. 4% globin	390	205	205
800 ml. 4% globin	650	235	250
800 ml. 4% globin	410	387	421
1,200 ml. 4% globin	330	180	310
1,800 ml. plasma	637	347	364
1,000 ml. plasma	567	524	496

other days he received 800 and 1,000 ml. of plasma, respectively. Each had a total protein concentration of 6.5 Gm. per 100 ml. The changes in plasma volume are seen in Table I.

Here it is apparent that the volume of retained globin solution varies depending upon the time elapsed and the quantity given. The same is true of plasma. Immediately after the infusions of globin and at two and at four hours, an average of 64 per cent, 36 per cent, and 40 per cent, respectively, of the globin was retained. These figures give oncotic values for 1 Gm. of globin at these three times of 16, 9, and 10 ml. of water, respectively.

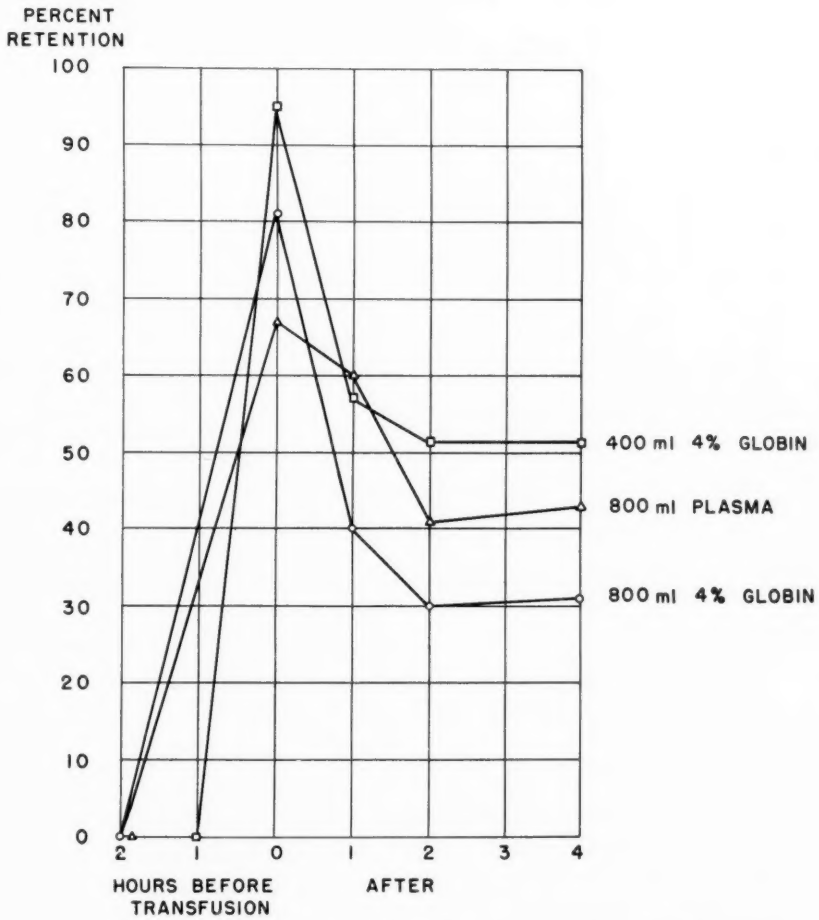

Fig. 4.—Effect of volume of transfusion on per cent retention.

With the use of plasma, the average gain in plasma volume immediately after the transfusions and two and four hours after, expressed in per cent of the injected material, was 68 per cent, 47 per cent, and 47 per cent. These figures give oncotic values for a gram of "plasma protein" of 10.7, 7.1, and 7.1 ml. of water, respectively.

The figures for oncotic values for globin and plasma proteins in this particular subject are less than those generally attributed to these substances. It must be noted that these observations were made in individuals with an essentially normal blood volume, and illustrate how difficult it is to determine such values with any degree of accuracy. Any biological experiment is subject to errors from uncontrollable and sometimes unrecognized variables. The volume of material injected, for example, seems to bear a definite relationship to the volume retained. A smaller volume shows a higher proportional retention, within certain limits, than does a large amount. The state of the recipient's plasma volume also undoubtedly influences the amount of material retained. If the original blood volume is smaller than normal, it is reasonable to assume that a larger proportion of any infusion will be retained.

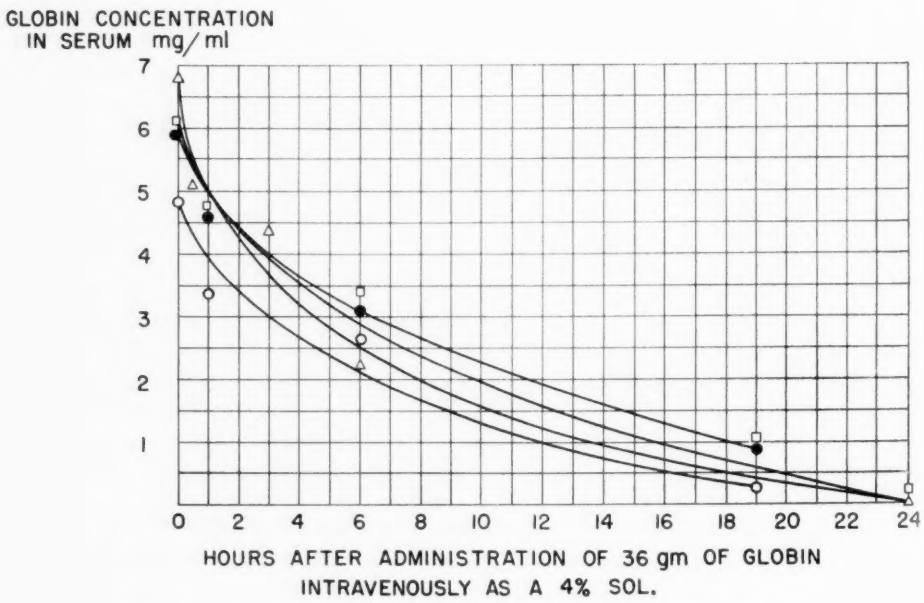

Fig. 5.—Rate of disappearance of globin from the circulation.

Fig. 4 illustrates how, in a single individual, a larger proportion of a 400 ml. infusion is retained than of an 800 ml. infusion. In this individual, the initial measured blood volume deficit, from the expected normal, was 700 ml.

Disappearance of Globin from Circulation.—Numerous observations have been made on the disappearance of transfused globin from circulation. Four typical records are presented graphically in Fig. 5. Generally not more than 60 per cent of the injected globin has been detected in the circulating plasma even when the examination has been carried out immediately after transfusion and when the plasma volume increase closely corresponds to the volume of injected globin solution. The average amount of globin present one hour after

completion of the transfusion is approximately 50 per cent of the total injected. As a rule no globin, or only a trace, remains in circulation after twenty-four hours, even when the increase in the circulating plasma volume persists longer.

The above observations were made on individuals who had received 36 Gm. of globin as a 4 per cent solution. The 900 ml. volume was administered in approximately two hours.

When a smaller amount of globin is given, a larger proportion of the material is retained in the plasma. Fig. 6 represents the proportions of globin retained in an individual who received 4.75 Gm. of globin in 50 ml. of saline in one and one-half minutes; and in another who received 36 Gm. in 900 ml. in one hundred twenty minutes. The small quantity of rapidly administered material was 80 per cent retained; the larger, more slowly administered material, was only 50 per cent retained immediately after the termination of the infusion.

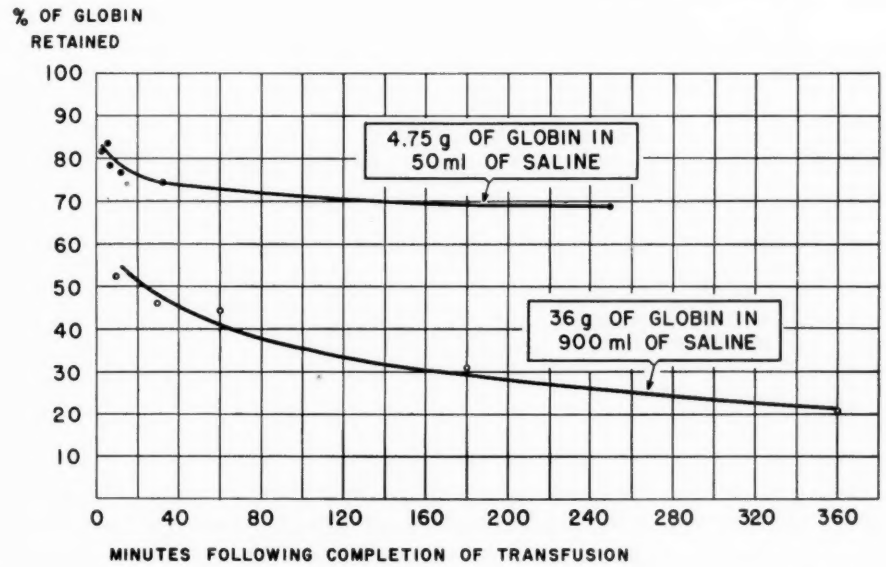

Fig. 6.—Effect of quantity of globin injected on the per cent retention in the blood stream.

TABLE II. DISPROPORTION BETWEEN THE AMOUNT OF GLOBIN RETAINED IN CIRCULATION AND THE INCREASE IN THE PLASMA VOLUME

	PLASMA VOLUME	TOTAL GLOBIN IN PLASMA	TOTAL CHANGE IN PLASMA VOLUME	VOLUME CHANGE ACCOUNTED FOR BY RETAINED GLOBIN*
Pretransfusion	2105			
Immediately after transfusion	2515	6.6	+410	+185
1 hour	2493	5.2	+388	+130
2 hours	2493	3.6	+388	+ 90
3 hours	2385	3.8	+280	+ 92
5 hours	2526	3.3	+421	+ 81
22 hours	2313	0	+207	0

Total twenty-four-hour globin loss in the urines: 3.43 Gm.
*On the basis of an optimal iso-oncotic value of 4 per cent.

TABLE III. LOSS OF GLOBIN IN THE URINE IN TWELVE PATIENTS RECEIVING DAILY 32 TO 44 GM. OF GLOBIN INTRAVENOUSLY AS A 4 PER CENT SOLUTION

	TOTAL GLOBIN IN URINE	PER CENT OF INJECTED GLOBIN LOST IN THE URINES
Average	2.63 Gm./24 hours	6.9
Maximum	4.0 Gm./24 hours	10.0
Minimum	0.52 Gm./24 hours	1.2

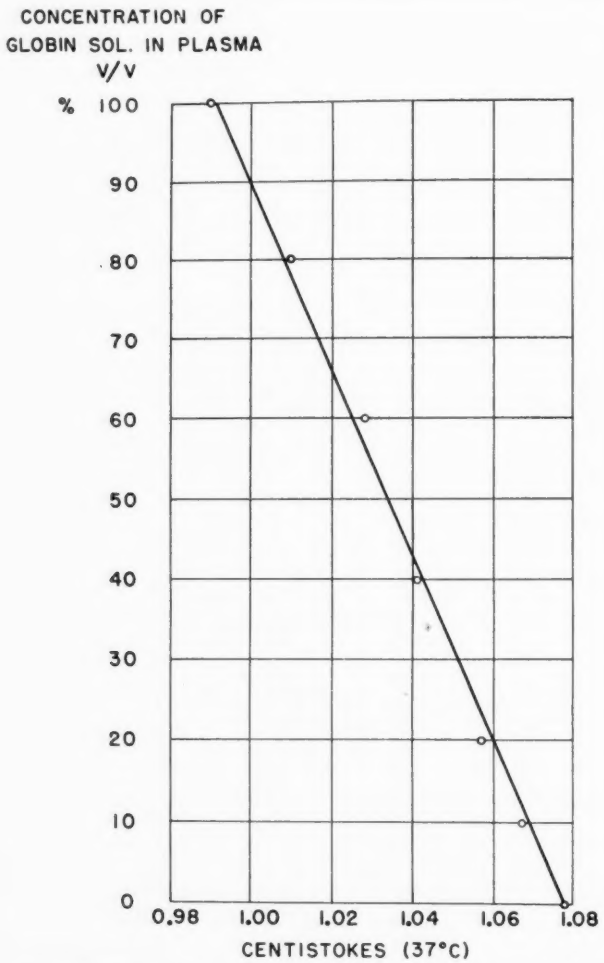

Fig. 7.—Effect of addition of globin solution (3.94 per cent) to plasma (6.1 per cent protein) on the viscosity.

A disproportion between the amount of globin retained and the increase in the plasma volume has been noted in practically every case. A typical example follows:

A 52-year-old man received 32 Gm. of globin as a 4 per cent solution over a period of about two hours. Immediately after transfusion, and at intervals of time, thereafter, determinations of the globin present in the serum and changes in the plasma volume were made. Results are shown in Table II.

Excretion of Globin in the Urine.—In all the observations carried out, globin loss in the urine has been small. In twelve patients receiving from 32 to 44 Gm. of globin as a 4 per cent solution intravenously, the loss of globin in the urine is shown in Table III. Patients retaining globin in the serum longer do not necessarily eliminate more globin in the urine. Even with rapid administration of very large doses of globin, the loss in the urine remains relatively small. A patient suffering from very extensive body burns received 160 Gm. of globin over a period of eight hours. In addition she received large amounts of plasma. In the twenty-four hours following the transfusion the patient had a urinary output of 1,220 ml. containing 17.8 Gm. of globin (11.1 per cent of the amount injected). In the urine of the next twenty-four hours, no globin appeared.

TABLE IV. EFFECT OF TRANSFUSION OF GLOBIN AS A 4 PER CENT SOLUTION IN A WOMAN SUFFERING FROM CHRONIC GLOMERULONEPHRITIS WITH EXTENSIVE EDEMAS

DATE	PLASMA VOLUME	SERUM PROTEIN (GM. %)	TOTAL PLASMA PROTEIN (GM. %)	BODY WEIGHT (KG.)	GLOBIN TRANSFUSED (GM.)	GLOBIN ELIMINATED IN THE URINE IN 24 HOURS		URINE 24 HOURS (ML.)	TOTAL N INTAKE	TOTAL URINARY N OUTPUT (GM.)
						TOTAL GM.	% OF GLOBIN ADMINISTERED			
Feb. 16	2269*	5.4	122	68.1				600	9.6	5.2
17					50	2.7	5.4	1950	10.9	10.5
18					50	4.0	8.0	1000	16.3	9.8
19					50	3.6	7.3	790	15.7	7.3
20					50	4.1	8.1	1425	13.3	8.5
21					50	1.8	4.5	900	15.2	5.3
22	2518	6.1	153	64.5						
					Average	3.2	6.6	1213	14.2	8.3

*Expected normal, 2490 ml.

Kidney damage, with proteinuria, in the course of chronic glomerulonephritis does not necessarily increase the rate of globin loss in the urine. A 35-year-old white woman, suffering from recurring glomerulonephritis, with hypoproteinemia and generalized edema, received for each of five days 50 Gm. of globin as a 4 per cent solution intravenously. The data on globin intake and urinary losses and other pertinent data are contained in Table IV. This patient had a concentration of globin in the serum of 8.8 mg. per milliliter immediately after the first transfusion of 50 Gm., representing 40 per cent of the injected material. Within seventeen hours no globin was present in the serum. She lost 2.7 Gm., or 5.4 per cent of the injected globin in the urine within twenty-four hours, while passing an average of 10 Gm. of albumin a day. This patient appeared to have retained the bulk of the globin injected. She had a weight loss of 2.7 kg., no evidence of urea retention, nor increase in the urinary N output for several days after discontinuance of globin.

Viscosity Studies.—Globin has a viscosity considerably less than that of plasma. When in vitro mixtures of globin and plasma are studied, the vis-

cosity diminishes with increasing globin concentrations (Fig. 7). However, when globin is injected in a patient and the viscosity of samples of plasma is tested, the viscosity is found to increase rather than decrease with the higher globin concentrations. This observation requires further study.

DISCUSSION

Ideally a plasma substitute, or extender, should, when injected in adequate amounts, completely correct existing deficits in blood volume, and the excess should fairly promptly diffuse out of the vascular spaces. The rapidity of such diffusion is subject to many variables, but excessive retention in circulation would cause hypervolemia and undesirable circulatory overload. Equally undesirable is a material which is lost in excessive amounts in the urine, such as gelatin, or which is retained in tissues as a foreign material, such as gum acacia, pectin, and, in part at least, polyvinyl pyrrolidone. A plasma substitute should not act as an antigen and, ideally, should be metabolized. Modified human globin appears to fulfill well the requirements stated above.

Previous reports[1-5] have dealt with the antigenic properties of globin, the utilization as a source of nitrogen, and the diuretic effect.

The experimental data given in this paper deal especially with the variations of the plasma volume, the rate of disappearance of injected globin from the circulation, and the amount lost in the urine. The amount of water held in circulation by injected macrocolloidal solutions and the duration of hemodilution vary considerably according to the nature of the material injected and the state of the recipient.

On the basis of maximal retention (Figs. 1 and 2), globin appears to be iso-oncotic in approximately 4 per cent solution. When globin is injected in patients with a known diminished plasma volume due to an acute loss, such as hemorrhage or burns, and in quantities not exceeding the patient's deficit, the hemodilution is generally close to the expected. However, in subjects with a normal volume of plasma, the measured maximal increase in the volume following the transfusion is generally much less than expected. The loss is affected by many variables, such as the speed of injection, the state of extravascular fluids, the cardiac condition, and, of course, the nature as well as the volume of the fluid injected. These unpredictable variables are reflected in the variations of hemodilution shown in Fig. 3. In this experiment the amount of globin injected in the eight patients was calculated to produce a 25 per cent increase in the plasma volume; actually the increases measured between 11 and 27½ per cent. The same figure shows that the hemodilution in the hypoproteinemic patients with a diminished volume of circulating plasma may last more than twenty-four hours. In a normal patient the blood volume is generally back to normal within twenty-four hours.

The amount of globin solution injected has a great deal to do with the proportion retained, an effect which can be readily expected. Fig. 4 shows the

hemodilution obtained in the same subject from the administration of 400 and 800 ml. of 4 per cent globin, expressed in per cent retention of the injected material.

When the data of hemodilution obtained with normal human plasma are compared with those obtained with a 4 per cent globin solution under similar conditions, it is apparent that hemodilution from plasma is similar but appears generally to last for a somewhat longer period of time.

In analyzing data on the rate of disappearance of globin from the circulating blood and the variations in the blood volume of normal patients, it appears that ordinarily no more than 60 per cent of the injected globin is present in the plasma even when the sample is taken immediately after the transfusion. This is also true even when measurements of plasma volume indicate retention of the entire volume of the infusion. This is taken to indicate a rapid exchange between the globin in the circulating plasma and the plasma proteins of the extravascular spaces. It is only when small amounts of globin are injected rapidly that a major portion of the material can be identified in the plasma in higher concentration for a period of at least a few hours.

SUMMARY

1. Solutions of approximately 4 per cent globin in 5 per cent dextrose or 0.85 per cent sodium chloride produce increases in plasma volume equivalent to 50 to 100 per cent of the volume of the injected material.

2. The increase of plasma volume in a recipient is influenced by the normalcy of his own plasma volume and the volume infused. When the plasma volume is near normal a smaller infusion shows a larger proportional retention than a larger infusion.

3. The "oncotic value" or the amount of water held in the circulation by 1 Gm. of globin appears to be between 12 and 25 ml. The determination in any one individual is influenced by his plasma volume and probably by many other factors.

4. The concentration of globin in a recipient's plasma is always less than would be expected if all of the material remained in the plasma in an unaltered form. Immediately after an infusion of 1,000 ml. of 4 per cent globin over a period of one to two hours, the plasma levels show generally only 50 to 60 per cent of the material injected. If a smaller amount of globin is injected rapidly, the plasma levels indicate that 80 per cent is present in the circulation.

5. Blood levels of globin drop in a curvilinear fashion over a period of six to twenty-four hours.

6. From 1.2 to 11.1 per cent of injected globin may appear in the urine within twenty-four hours.

7. The viscosity of 4 per cent globin is less than plasma. The viscosity of in vitro mixtures of 4 per cent globin and plasma is less than the viscosity of plasma alone. Preliminary observations indicate that these findings are reversed in vivo, so that the plasma of a patient receiving globin has a slightly higher viscosity.

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BRONCHOSPIROMETRY

III. Complications, Contraindications, Technique, and Interpretation

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BECAUSE the bronchospirometric technique has undergone a number of modifications in an effort to avoid complications, it is perhaps appropriate to follow an unusual order of presentation by first discussing these complications.

COMPLICATIONS

Material for Analysis.—One thousand brochospirometric examinations have been performed at our laboratories during the past four years. This entire experience could not be used to determine the incidence of each complication. The procedure was performed under topical anesthesia on 860 occasions, and this number is available for analysis of reactions to these locally applied agents. To determine the effect of the procedure on the subsequent status of pulmonary tuberculosis, a definite follow-up period was required. Collapse or resection was performed less than one week after bronchospirometry in 290 patients; the postoperative course could not be obtained in ten patients who were not seen again, and 140 of the remaining group did not have tuberculosis. The subsequent status of pulmonary tuberculosis could be examined in the remaining 420 patients.

Reactions to Local Anesthetic Agents.—The entire gamut of local anesthetic substances has been used at some time to facilitate introduction of the double-lumen catheter. Two per cent tetracaine with or without epinephrine appears to have been the most widely used agent.[1-5] Larocaine,[6, 7] Nupercaine,[8] and Xylocaine[9] have been used alone and in various combinations with cocaine.[10-12] Our initial studies were made using Forrestier's solution, a preparation of cocaine, phenol, and epinephrine in a cherry laurel water base, as recommended

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TABLE I. COMPLICATIONS OF BRONCHOSPIROMETRY

COMPLICATION	PREMEDICATION AND TOPICAL ANESTHESIA*								
	ATROPINE, MORPHINE AND FORRESTIER'S SOLUTION			TETRACAINE ONLY			TOTAL		
	TOTAL NO. OF PATIENTS	NUMBER COMPLICATED	PER CENT	TOTAL NO. OF PATIENTS	NUMBER COMPLICATED	PER CENT	TOTAL NO. OF PATIENTS	NUMBER COMPLICATED	PER CENT
Toxic response to topical anesthetic in "normal" individuals	107	0	0	753	2	0.3	860	2	0.2
Allergic response to topical anesthetic in patients with bronchial asthma	5	2	40	27	12	44	32	14	44
Fibrile reaction in patients with pulmonary tuberculosis	97	46	48	333	71	21	420	117	28
"Spread of Disease" in patients with tuberculosis	97	8	8.3	333	1	0.3	420	9	2.1
Fatal exacerbation of disease in patients with tuberculosis	97	1	1.0	333	0	0	420	1	0.1

*All patients were premedicated with short-acting barbiturate.

by Wright and Michelson.[13] This solution provided reliable and long-lasting anesthesia, and no untoward reactions were encountered (Table I). Its routine use was abandoned because the greatly prolonged anesthetic action was felt to have a deleterious effect on the disease in patients with profuse secretions and in patients with pulmonary tuberculosis. It is still employed in normal volunteers and in patients where prolonged procedures are planned.

Recently, there has been a trend toward use of topical anesthetic agents in more dilute solutions.[14-17] Greater dilution permits use of larger quantities of fluid without exceeding the accepted total dosage, and a more uniformly distributed surface coverage is possible. This mode of application also appears to be safer because it is thought that the toxicity of local anesthetic agents increases in geometrical, rather than arithmetical, progression with increasing concentration.[18]

Accordingly, we have used 0.25 per cent tetracaine for endotracheal injection as suggested by Harken and Salzberg[19] and by Corabelli.[20] This dilute solution provides adequate anesthesia of short duration. The early return of the cough reflex and ciliary motion are thought to be decidedly advantageous, particularly in patients with tuberculosis.

Barbiturate premedication, a well-accepted preliminary to topical anesthesia, has been employed throughout the series. All hospitalized patients received 0.1 Gm. of pentobarbital at bedtime and again one hour before operation.

If we adopt the classification of reactions to local anesthetic agents proposed by Sadove and associates,[18] we find that two "normal" individuals in our series showed a toxic response in the form of central nervous system stimulation, an incidence of 0.2 per cent (Table I).

CASE REPORTS.—A 48-year-old man with pulmonary tuberculosis received twice the usual amount of tetracaine because of an unusually persistent and forceful cough reflex. After the last endotracheal instillation he began to show slight rhythmical movements of all four extremities. He remained seated, and continued to answer questions confusedly. After three minutes the movements diminished and the bronchospirometric catheter was introduced. The examination proceeded uneventfully, although slight twitching of hands and feet continued for about ten minutes.

The second patient, a 27-year-old man, was studied after bilateral thoracoplasty. Shortly after initiation of topical anesthesia with 0.25 per cent tetracaine he developed a marked nystagmus, lost consciousness, and exhibited mild generalized convulsive movements. Recovery was rapid after intravenous administration of thiopental. An unusually large amount of tetracaine may have been absorbed because the patient was unable to cough and most of the solution was retained in the lungs.

Similar reactions, attributed to topical anesthesia prior to bronchospirometry, have been reported by others. Vaccarezza and his group,[1] using 2 per cent tetracaine, recorded evidence of toxicity in 0.5 per cent of patients; Björkman[4] noted untoward reactions in 8.6 per cent of his first seventy patients, and Pinner and associates[11] noted that reactions to cocaine were common.

Among "abnormal individuals" there were many "allergic responses" according to the classification by Sadove and associates.[18] Of thirty-two patients in our series with a history of bronchial asthma, fourteen experienced attacks during preparation with the topical anesthetic. The incidence of these

episodes was the same whether Forrestier's solution or tetracaine was used (Table I). Respiratory studies of one asthmatic before and after preparation with tetracaine have been reported.[21] Most patients responded well to parenteral or aerosol administration of bronchodilator drugs, and in no instance was the episode so prolonged or so severe as to cause abandonment of the examination. Since 44 per cent of asthmatics reacted in this manner, we now observe special precautions in dealing with them. Whether the topical anesthetic itself is responsible for precipitation of the attacks is conjectural. Most of these patients had had tetracaine for bronchoscopy or bronchography so that sensitization may well have taken place. In several instances the onset of an attack was noted upon arrival at the bronchospirometry room even before the anesthetic had been administered. The thought of the pending procedure and the sight of the apparatus may have been a sufficient psychic stimulus for precipitation of an attack.[22]

We have attributed the infrequency of minor reactions and the absence of serious reactions to the sparing use of very dilute solution of tetracaine, the routine use of barbiturate premedication, and certain other precautionary measures which will be enumerated subsequently.

Local Trauma.—Some sequelae due to local trauma by the catheter appear after almost every intubation. Hoarseness and difficulty in swallowing, a slightly painful throat, and a moderate increase in cough and expectoration, persisting for the remainder of the day, were encountered after nearly every intubation. These reactions were more severe after unusually prolonged procedures in very ill patients in whom intubation was difficult. Bronchoscopy has occasionally been performed immediately after the procedure. Slight reddening of the trachea and left main bronchus was invariably observed.

Effect of Bronchospirometry on the Subsequent Status of Pulmonary Tuberculosis.—Ever since broncoscopy and bronchospirometry were first performed in patients with pulmonary tuberculosis, there have been misgivings in the minds of both the patients and the physicians regarding the possibility of aggravation of the disease. Although the effect of bronchospirometry has been evaluated only once in this regard,[23] a number of studies are available dealing with analysis of the effect of bronchoscopy.

Warren, Hammond, and Tuttle[24] recorded extension of disease following bronchoscopy in one per cent of 198 cases. Hawkins,[25] reporting his experience with 516 patients, noted a mortality of 0.6 per cent and an extension of the disease in 0.2 per cent. McIndoe, Steele, and Samson[26] conducted a careful study of the course of the disease following 272 routine bronchoscopies. Roentgenograms were obtained one month before and three weeks after bronchoscopy, and temperatures were taken at four-hourly intervals for one week before and after bronchoscopy. An increase in the pulmonary disease was noted in 1[illegible] per cent, and 12 per cent showed a temporary temperature elevation. They concluded that a similar incidence of roentgenographic changes could be expected in any comparable group of patients with active tuberculosis who had not had bronchoscopy. Radner[27] observed a febrile response after bronchoscop[illegible]

in 10 per cent of 183 patients. The temperature elevations, which ranged from 1° F. to 3° F., were thought to be nonspecific reactions and of no clinical significance. Although none of the patients had shown recent progression or extension of the disease prior to bronchoscopy, definite roentgenographic changes consisting of atelectasis, fresh areas of infiltration, or increase in the size of cavities were noted in 14 per cent. These adverse results were attributed to abolition of the cough reflex and disturbed ciliary action following the procedure. The importance of the use of short-acting barbiturates, avoidance of intense tracheal anesthesia, and encouragement of cough were advocated as prophylactic measures. MacRae and associates[28] showed that patients who had exhibited previous spreads were much more prone to show further extension after bronchoscopy. From comparison of roentgenograms taken three months before and three months after the procedure, they concluded that bronchoscopy is responsible for dissemination of tuberculosis chiefly in patients who have endobronchial involvement. Coates[29] reported a detailed statistical analysis of the results of 473 bronchoscopies. Roentgenograms were taken at two-monthly intervals for six months before and six months after bronchoscopy and were the sole basis for evaluation. Evidence of increase of disease in the two-month period after bronchoscopy was found in 15 per cent. However, the same or a larger incidence of increases had occurred during all three two-month intervals prior to bronchoscopy. Although it was admitted that reactivation must be occasionally attributed to the procedure on clinical grounds, it was concluded that there was no evidence from a statistical standpoint to support these clinical impressions.

The course of tuberculosis following bronchospirometry has never been analyzed. Of the first seventy patients submitted to the procedure by Björkman, one-half showed a rise in temperature, but this was apparently not accompanied by extension of the disease.[4] Arnaud[30] and Pinner[11] both reported one death due to tuberculous pneumonia. Occasional extension of disease has been mentioned, although the actual incidence was usually not stated.[31] Wright and Michelson[13] have observed "reactivation of quiescent disease, spread of disease to new locations, and exacerbations of the existing disease." These sequelae occurred in patients with tuberculosis in its most unstable form. They thought that bronchospirometry had no significant effect on the natural course of disease. Their impressions were gained from data analyzed in the manner of Coates,[29] which have not yet been reported, showing a lower incidence of increased activity in the post-test period than in the pre-test period.

Statistical analyses of this kind may suggest that the course of tuberculous disease in large groups is not altered by bronchial intubation. This is of little assurance to the individual patient. By the same type of reasoning one could, for example, compare the clinical course of patients with far-advanced tuberculosis. If one group were not treated and another treated with pneumonectomy, and if the same number of patients in both groups were alive six months later, one might erroneously conclude that pneumonectomy carries no operative mortality, or that it does not alter the course of the disease.

TABLE II. FEBRILE REACTION AND EXACERBATION OF DISEASE FOLLOWING BRONCHOSPIROMETRY IN PATIENTS WITH PULMONARY TUBERCULOSIS

EXTENT OR SEVERITY OF DISEASE	PREOPERATIVE AND POSTOPERATIVE TREATMENT									
	FORRESTIER'S SOLUTION, MORPHINE, ATROPINE					DILUTE TETRACAINE, POSTURAL DRAINAGE, PROPHYLACTIC PENICILLIN				
	TOTAL GROUP OF 97 PATIENTS		TEMPERATURE ELEVATION TO 100.8° F.	TEMPERATURE ELEVATION OVER 100.8° F.	SPREAD OF DISEASE	TOTAL GROUP OF 333 PATIENTS		TEMPERATURE ELEVATION TO 100.8° F.	TEMPERATURE ELEVATION OVER 100.8° F.	SPREAD OF DISEASE
	NUMBER	PER CENT	PER CENT	PER CENT	PER CENT	NUMBER	PER CENT	PER CENT	PER CENT	PER CENT
Apparently arrested or minimal disease (I)	20	20	21	0	0	81	24	20	0	0
Moderately advanced Disease (II)	23	24	60	8	13	60	18	30	2	0
Far-advanced disease (III)	54	56	31	19	9	192	58	20	2	0.5
No symptoms (A)	22	23	23	0	0	91	27	23	0	0
Slight symptoms (B)	23	24	35	4	13	68	20	25	0	0
Moderate symptoms (C)	41	43	46	12	0	142	43	17	2	0.7
Severe symptoms (D)	10	10	30	50	50	32	10	34	3	0
Total incidence of fever and spreads	97	100	36	13	8.3	333	100	22	2	0.3

A program was therefore outlined which, we hoped, would give an indication of the effect of bronchospirometry on the course of the disease in any individual patient. A roentgenogram of the chest was taken the day before bronchospirometry, one week later and, whenever possible, six weeks afterward. A special four-hour temperature chart was kept for one week, and marked increase of cough or expectoration, streaking or frank hemoptysis, hoarseness, or unusual pain were recorded.

The incidence of febrile reactions and the incidence of extension of disease of 420 patients examined in this manner are shown in Table II. The extent and symptomatology of the disease were classified according to the criteria of the National Tuberculosis Association.

The first ninety-seven patients received 10 to 16 mg. of morphine and 0.4 to 0.6 mg. of atropine one-half hour before operation, and Forrestier's solution was used as topical anesthetic. A febrile reaction occurred in 49 per cent shortly after bronchospirometry and the temperature was elevated by more than 2° F. in 13 per cent (Table II). The reactions were more frequent and more serious in patients with far-advanced disease and in those with moderate or severe symptoms. The patients, as a group, were rather ill. Twenty-four per cent had moderately advanced, and 56 per cent far-advanced tuberculosis. The patients included in groups "III" as well as "D" had tuberculosis in its most unstable form. They represented 10 per cent of the group and of these, 80 per cent showed a febrile reaction after the procedure and the temperature elevation was over 2° F. in 50 per cent. All five patients in this category who had marked temperature elevation also showed a definite exacerbation of disease on roentgenograms taken one week after the procedure. There were three other patients in the group medicated with atropine and morphine who showed an increase of disease (Table II). The total rate of spread was 8.3 per cent, and one death was attributed to bronchospirometry (Table I).

Case Report.—P. O'D., a 22-year-old male clerk, was admitted sixteen months prior to the examination with tuberculous pneumonic infiltration throughout both lungs and multiple cavities in the left upper lobe. Severe diabetes mellitus was poorly controlled at the time of admission. Treated with strict bed rest, pneumoperitoneum, 3 Gm. of streptomycin a day for four months, a regulated diet, and 110 units of insulin a day, he improved slowly. He continued to cough and raise large amounts of sputum. Occasionally evening temperatures were elevated to 99.6° F. A roentgenogram made the day before bronchospirometry showed minimal fibrotic changes on the right, but the entire left upper lobe was excavated. Following uneventful bronchospirometry with morphine and atropine premedication and Forrestier's solution locally, the patient felt well, although his temperature rose to 100.2° F. that night. Streptomycin and penicillin therapy was started, and additional insulin was required. During the next four days morning temperatures were normal, and evening temperatures ranged from 100.0° F. to 100.6° F. The pulse rate increased from 80 to 160, and respirations from 18 to 44. The fasting blood sugar was 356 mg. per cent on the second day, and 460 mg. per cent the fourth day. Dyspnea became severe in spite of oxygen therapy. A portable roentgenogram revealed a pneumonic process involving the entire right lung. The patient died on the fifth day in spite of bronchoscopic aspiration and intravenous feedings. Over 400 units of insulin were administered during the last day of his life. Permission for post-mortem examination could not be obtained. A combination of respiratory insufficiency and diabetic coma was thought to have been the immediate cause of death.

Exacerbations have not been limited to patients with far-advanced and very unstable disease.

M. McK., a 27-year-old woman, was not as ill as the previous patient. On admission, two years prior to bronchospirometry, two small cavities were demonstrated, one at the apex of each lung. With pneumoperitoneum treatment the patient made good progress. A chest film made the day before bronchospirometry showed the lung fields essentially clear except for a small residual cavity and infiltration at the right apex (Fig. 1, *A*). Bronchospirometry, performed with morphine and atropine premedication and Forrestier's solution anesthesia, showed only minimal functional impairment of the right lung. This patient had been without cough or sputum for one year, and her temperature had never been elevated. Eight hours after bronchospirometry the temperature rose to 99.8° F. and increased to 102.8° F. on the second day and to 104° F. on the third day. This was accompanied by the sudden appearance of productive cough, anorexia, malaise, and night sweats. Streptomycin treatment was begun,

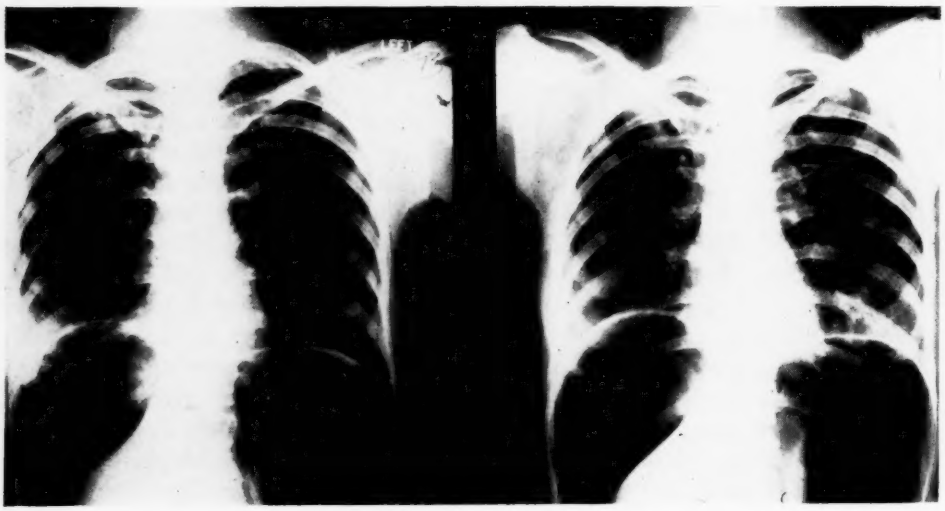

Fig. 1.—Chest roentgenograms of patient M. McK. one day before and one week after bronchospirometry. Initially a small cavity was present at the apex of the right lung. After the procedure a "spread" has occurred to the lower portion of the left lung.

and the evening temperature slowly returned to normal during the next twenty-eight days. A roentgenogram made one week after bronchospirometry showed a new area of infiltration at the base of the left lung (Fig. 1, *B*). This diminished with time, but never entirely disappeared. A selective apical thoracoplasty was performed two months later. The postoperative course was uneventful, and she has been well and doing full-time work for the past two and one-half years.

It is believed that the occasional unfavorable effect of bronchospirometry on the course of the disease is not due to the intubation itself. We agree with Amberson[32] and Radner[27] that such incidents are due to retention of purulent material and implantation of bacillus laden secretions in previously unaffected parts of the lung. Premedication and topical anesthesia both favor such retention. Barbiturates often cause sleep for some hours after intubation, atropine makes the secretions more viscid and therefore difficult to raise, morphine acts as a potent anti-tussive, while Forrestier's solution deprives the patient of endobronchial sensation for one to two hours.

A new regimen, designed to alter as many of these factors as possible, was therefore instituted. Premedication with morphine and atropine was omitted entirely. The topical anesthetic was changed to the shorter acting tetracaine which was used in very dilute solution. Only the use of barbiturates was continued because they afford protection against toxic reactions to the topical anesthetic. Postural drainage was performed immediately after operation, and repeated every three hours for the remainder of the day and three times a day for three days. Patients were not permitted to sleep during the immediate postoperative period. Parenteral penicillin was given prophylactically for three days or until the temperatures had been normal for three days. An attempt was made to exclude tuberculous patients such as those in group "III D."

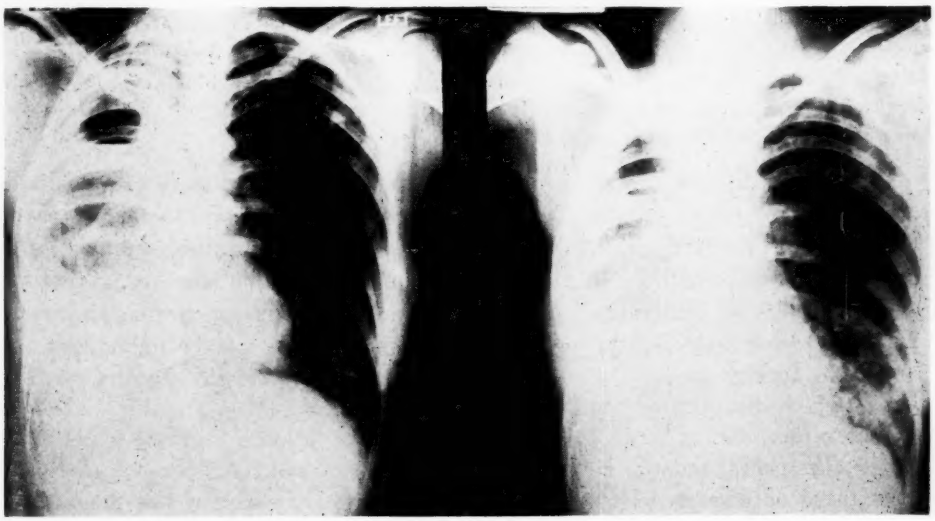

Fig. 2.—Chest roentgenograms of patient M. McG. one day before and one week after bronchospirometry. The procedure was followed by new tuberculous involvement of the lower portion of the left lung.

The results with this new regimen following 333 bronchospirometries are shown in Tables I and II. Although rapidly progressive tuberculosis in very ill and toxic patients was now considered a relative contraindication to bronchospirometry, the severity of the disease in this second group remained unaltered. Only 24 per cent had apparently arrested or minimal tuberculosis, and 58 per cent had far-advanced disease. Again, 10 per cent of the entire group were classified as having far-advanced disease with serious symptoms (Group "III D"). Nevertheless, by this new regimen, the incidence of febrile reactions was reduced from 49 to 24 per cent, and the incidence of elevations of more than 2° F. was reduced from 13 to 2 per cent. Again, as in the first group, patients with minimal or apparently arrested disease and patients without symptoms showed the fewest febrile reactions (Table II). There was only one who showed exacerbation of disease; the "spread" rate was reduced from 8.3 to 0.3 per cent (Tables I and II).

It is felt that, with the revised regimen, bronchospirometry can be performed safely even in patients who are seriously ill with tuberculosis. The importance of a vigorous cough reflex, effective ciliary motion, and adequate mobilization of secretions was emphasized by the greatly improved results in the second group where only one spread occurred following 333 intubations:

CASE HISTORY.—M. McG., a 36-year-old woman, was readmitted to the Sanatorium Division of the Boston City Hospital three months before bronchospirometry. The roentgenogram the day before bronchospirometry showed excavation of the entire upper lobe of the right lung and a lower lobe which had only partly re-expanded after abandonment of therapeutic pneumothorax two years before (Fig. 2, *A*). The left lung was overdistended. A small area of infiltration overlying the second anterior rib had remained unchanged for two years. Bronchospirometry, performed according to the newly revised regimen, was followed the next morning by a temperature of 99.6° F., and rose to 102.8° F. that evennig. Streptomycin therapy was started, and within the next ten days the temperature returned to the preoperative level. A roentgenogram taken one week after bronchospirometry showed a diffuse spread to the lower part of the left lung (Fig. 2, *B*). Streptomycin was continued for two months, and a right pneumonectomy was then performed. The postoperative course was uneventful, and the patient has been well at home for two years.

Undoubtedly, many of the febrile reactions were not due to exacerbation of existing disease or implantation of organisms in previously unaffected parts of the lung. Early appearance of the fever suggests "autotuberculinization" or liberation of foreign protein material as the source of the reaction. Whether the new areas of infiltration observed on the chest roentgenograms one week after operation were due to new foci of disease or were merely pulmonary reaction to foreign protein material must remain unanswered. In the past, a lesion which disappeared within a few weeks without leaving a residual shadow has been regarded as being due to "autotuberculinization," and one which persisted for several months and healed by scar formation has been considered a new focus of disease. However, truly new foci of disease in the pneumonic stage, when treated with streptomycin, may also disappear within a few weeks without leaving radiopaque residua. Early streptomycin treatment therefore makes this differentiation very difficult. The persistence of lesions, in spite of streptomycin treatment, in the two patients whose course was described, suggests that the roentgenographic shadows represented disease in previously unaffected portions of the lung.

Other Complications.—Blood-streaked sputum or frank hemoptysis have been described following bronchospirometry. This was not seen in our series, possibly because we did not accept patients who had had evidence of bleeding during the two weeks preceding the operation. Chills and transient high fever were seen on a few occasions in patients with and without tuberculosis. These subsided within a few hours, and may have been due to transient bacteremia. Similar reactions are seen after other endoscopic procedures, particularly cystoscopy. In patients with bronchial asthma, attacks occasionally began after introduction of the catheter. This was never severe enough to cause interruption of the procedure. In fact, bronchospasm was purposely induced a number of times during bronchospirometry by intravenous injection of methacholine or histamine.

CONTRAINDICATIONS

The contraindication to bronchospirometry which has been mentioned most frequently is tuberculous ulceration of the trachea, larynx, or left main bronchus.[1, 3, 6, 8, 11, 13, 16, 17] The only other contraindication, recorded with some unanimity, is recent hemoptysis.[1, 11, 13, 16, 33] The procedure has been thought to be undesirable in the presence of a deformed tracheobronchial tree,[1] obstruction of the left main bronchus,[3, 6] high fever,[11, 16] the presence of viscid sputum,[11] and severe bronchiectasis.[34]

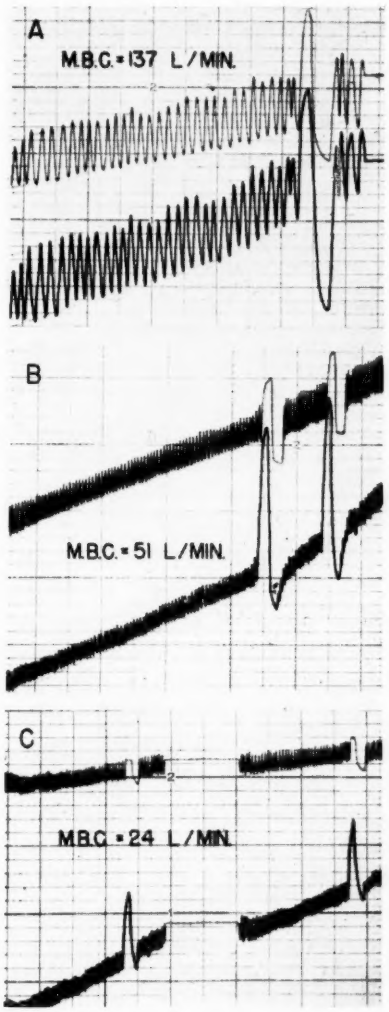

Fig. 3.—Bronchospirograms of three patients with varying degrees of ventilatory insufficiency. The first patient (*A*) with a maximum breathing capacity of 137 liters per minute had only slight ventilatory insufficiency and was not incapacitated. The second patient (*B*) had moderate ventilatory insufficiency and dyspnea during exercise. The third patient (*C*) with a maximum breathing capacity of 24 liters per minute was totally disabled with dyspnea on slightest exertion. Ventilatory insufficiency is not a contraindication to bronchospirometry. (In this and all subsequent tracings the respiratory excursions of the right lung are recorded above those of the left, the interval between the heavy horizontal rulings represents a volume of 500 ml., the interval between heavy perpendicular rulings, a time interval of one minute, and the spirograms read from left to right.)

Ulcerative tracheobronchial disease and hemoptysis within two weeks prior to planned bronchospirometry has been accepted by us as a routine contraindication. A deformed bronchial tree occasionally makes proper intubation difficult, and severe obstruction of the left main bronchus has prevented satisfactory bronchospirometry four times (Table III). Preoperative postural drainage, omission of atropine, and the use of catheters with lumina large enough to permit removal of secretions facilitate the procedure in patients with viscid sputum or bronchiectasis.

TABLE III. CAUSES OF UNSATISFACTORY RESULTS OF BRONCHOSPIROMETRY (860 PROCEDURES)

DIFFICULTY	NUMBER	PER CENT
Catheter could not be introduced into trachea	3	0.3
Catheter could not be introduced into left main bronchus		
a.) because of deformed tracheobronchial tree	3	0.3
b.) becauses of stenosis	4	0.5
Unsatisfactory tracing was due to:		
a.) Excessive secretions or uncontrollable cough	3	0.3
b.) Occlusion of orifice of left upper lobe on both tracings	9	1.0
c.) Air leak from one lung to the other or to outside on both tracings	3	0.3
d.) Catheter orifice for right lung was occluded by deviated trachea	15	1.7
Patient could not tolerate catheter because of dyspnea	1	0.1
Recourse to general anesthesia was necessary because of		
1.) Uncooperative patient (psychotic)	1	0.1
2.) Patient too young to cooperate	1	0.1
3.) Patient had unusual gag reflex	1	0.1
Technical difficulties (left to right leak within catheter; balloon developed leak after introduction; electrical short circuit; faulty spirometer valve; etc.)	5	0.6
Total unsatisfactory results	49	5.7

A low respiratory reserve has often been mentioned as contraindication to bronchospirometry.[3, 8, 11, 16, 23, 35] Some have thought that bronchospirometry should not be performed if the vital capacity is less than 1,500 ml.[23] Only Wright and Michelson[13] feel that this should not stand in the way of bronchospirometry. The three bronchospirograms reproduced in Fig. 3 illustrate the results of the examination in three patients with maximum breathing capacities of 137, 51, and 24 liters per minute, respectively. In our experience, no degree of ventilatory insufficiency has been severe enough to interfere with successful bronchospirometry. On the contrary, patients with low respiratory reserve have less ability to alter their functional residual capacity, and more easily interpreted bronchospirograms are obtained. No difficulty was encountered during bronchospirometry in patients after bilateral surgery. Even in patients with a very low respiratory reserve as encountered in pulmonary emphysema complicated by cysts or bullae, bronchospirometry was performed without mishap.

The data of Table IV suggest that dyspnea during bronchospirometry was not necessarily more severe in patients whose exercise tolerance was limited. Unusual shortness of breath during the procedure was experienced by only 1[illegible] per cent of all patients, although 32 per cent had a markedly limited pulmonary reserve. Comparison of the bronchospirometric vital capacity with the same

TABLE IV. RELATIONSHIP OF PREOPERATIVE DYSPNEA TO RESPIRATORY DISTRESS DURING BRONCHOSPIROMETRY AND TO THE VITAL CAPACITY EFFORT DURING BRONCHOSPIROMETRY

	TOTAL INCIDENCE OF PREOPERATIVE DYSPNEA (PER CENT)	RESPIRATORY DISTRESS DURING BRONCHOSPIROMETRY				COMBINED BRONCHOSPIROMETRIC VITAL CAPACITY AS PER CENT OF SPIROMETRIC VITAL CAPACITY (PER CENT)
		NONE (NO. OF PTS.)	SLIGHT (NO. OF PTS.)	MODERATE (NO. OF PTS.)	SEVERE (NO. OF PTS.)	
No preoperative dyspnea	66	736	43	7	10	81
Slight preoperative dyspnea (one flight of stairs)	9	14	8	1	6	77
Moderate preoperative dyspnea (walking on the level)	14	2	3	1	0	79
Severe preoperative dyspnea (on slightest exertion)	11	21	8	0	2	68
Total incidence of respiratory distress during bronchospirometry, per cent		90	7	1	2	

volume obtained during ordinary spirometry is a fairly good index of respiratory distress during intubation. This ratio was essentially the same in patients who ordinarily showed no, slight, or moderate dyspnea (Table IV).

The contraindications to bronchospirometry may be listed as follows:

I. Routine contraindications
   1. Ulcerative tracheobronchial tuberculosis
   2. Hemoptysis within two weeks.

II. Relative contraindications
   1. Obstruction of the left main bronchus
   2. Far-advanced pulmonary tuberculosis with severe symptoms and evidence of toxicity.

The last contraindication deserves further comment. If bronchospirometry is performed in patients with pulmonary tuberculosis in its most unstable form a small number of exacerbations must be expected. It is well to avoid the procedure in such patients unless the information is essential for clinical evaluation. Unfortunately, a large number of patients, in whom bronchospirometry is most urgently needed, fall in this very group. That our later hesitancy, in this respect, has not altered the type of patient admitted for bronchospirometry is illustrated by the data of Table II.

TECHNIQUE

Literature.—Two brief presentations by others have been devoted entirely to a discussion of the technique of bronchospirometry.[13, 16]

Preliminaries.—1. *Over-all function studies:* No patient should have bronchospirometry who has not had evaluation of over-all function. These preliminary studies should include at least vital capacity, maximum breathing capacity, some form of exercise test and recording spirometry, and preferably determination of functional residual capacity. Clinically, prior function studies are helpful because some patients show such poor respiratory reserve that plans for surgery must be abandoned, regardless of differential functional distribution. Other patients prove to have excellent pulmonary reserve, and almost any planned procedure can be undertaken without knowledge of the result of split function studies. From a research standpoint, bronchospirometry without knowledge of over-all function is not informative because it gives no indication of absolute values.[4, 11, 13, 16, 35] Finally, the local reaction to bronchospirometry is such that adequate over-all studies cannot be performed for some days afterward.

2. *Bronchoscopy:* Bronchoscopy is a desirable preliminary to bronchospirometry.[3, 6, 13] Technical problems, likely to be encountered during intubation, can be evaluated and the most important contraindication, ulcerative endobronchial tuberculosis, can be definitely established. No house patient at the Sanatorium Division of the Boston City Hospital or the Veterans Administration Hospital at Rutland has ever had bronchospirometry without prior bronchoscopy. Results of bronchoscopic examination were available in 94.5 per cent of our 1,000 studies.

3. *Special roentgenograms:* If any question exists concerning the configuration of the tracheobronchial tree or the patency of the left main bronchus, bronchograms are occasionally helpful. Laminograms have also been suggested as an aid for mapping out the tracheobronchial tree.[13]

4. *Preoperative treatment:* The night before operation the patient receives 0.1 Gm. of pentobarbital and the purpose of the procedure is explained briefly. On the morning of operation breakfast is omitted, and medication with a short acting barbiturate is repeated about one hour before the procedure. No further premedication is given to patients with tuberculosis, although most authors advocate large doses of atropine and morphine prior to

operation[1, 6, 8, 13, 30] with meperidine[5] and Pantopon[4] as alternatives. Proctor warned against atropine because it produces unwanted thickening of secretions, and Carlens suggested omission of morphine because of its respiratory depressing activity.[2]

In patients without pulmonary tuberculosis 6 to 10 mg. of morphine and 0.3 to 0.6 mg. of atropine are given subcutaneously shortly before operation. The importance of preoperative postural drainage has been emphasized.[6, 7, 13]

5. *Equipment and technical preparation:* The room selected for bronchospirometry should be quiet and must be equipped with a horizontal fluoroscope if a catheter other than the Carlens type is used.[31] Even with the latter tube we have preferred to check the position by fluoroscopy and by spot films in the operating room.[36] A screen with spot film attachment is preferable although adequate spot films of the catheter in situ can be obtained by centering the fluoroscopic screen over the target area. A small cassette is placed on the patient's neck and upper chest and the film is exposed for one to two seconds by depressing the foot switch for the fluoroscope.

Recording equipment should be amply tested prior to the patient's arrival. Twin spirometers of the Benedict-Roth type, arranged to record on one drum, are entirely satisfactory and are commercially available.* A modification of this apparatus designed for oxygen and ambient air recording spirometry will be described subsequently.[37] Differential ventilation and oxygen uptake may also be determined by the open circuit technique.[37]

In addition to the usual equipment required for administration of topical anesthesia, several bronchospirometric catheters are prepared out of sight of the patient. We have three catheters available for every procedure: a male and a female size Carlens catheter with stylets,[2]† and a Gebauer catheter,[6]‡ as modified by Wright.[13] All solutions, tetracaine, alcohol for sterilization, Forrestier's solution for prolonged anesthesia, or saline for dilution of the tetracaine should be color coded. Jackson forceps as modified by Wright[13] are helpful for introduction of the tube.

Alternative and Emergency Equipment.—A kit containing ampules of phenobarbital, thiopental, epinephrine, theophylline ethylenediamine, neosynephrine, and appropriate syringes, needles, and tourniquet is always on hand. A nebulizer, isopropyl-norepinephrine, syringes, and connecting tubing are prepared when asthmatics are to be studied. A suction machine is available with fine urethral catheters and plastic tubing for suction through the bronchospirometric catheters. A "trans-cricoid pack" contains 2 per cent procaine, syringes and needles for the rare uncooperative patient or patient with unusually sensitive gag reflex when topical anesthesia is administered by the trans-cricoid route described by Harken and Salzberg[19] and Magill.[38] An open-blade laryngoscope is used on rare occasions when intubation by direct laryngoscopy becomes necessary. Ideally, a thoracotomy kit and an anesthesia machine should be available, since toxic reactions to topical anesthetics occasionally manifest themselves by cardiac or respiratory arrest.

Topical Anesthesia.—The importance of adequate anesthesia cannot be overemphasized.[1, 7, 12, 13] Intubation should never be attempted unless complete anesthesia has been obtained. Bronchospirometry requires more thorough and careful anesthesia than either bronchoscopy or bronchography because the onset of cough may terminate the procedure prematurely.

The patient is seated and the plan of the procedure is outlined. He grasps his tongue with gauze, and palate, throat, and pharynx are lightly sprayed with 2 per cent tetracaine. Excessive solution is expectorated. The anesthetic can also be applied with a swab or brush.[12] Three to five minutes are then allowed for any possible reaction to occur in hypersensitive individuals. This time is used to check the spirometer pens and motor, to fill the spirometers with oxygen, and to test the system for tightness. After a second throat spray, cotton pledgets soaked with 2 per cent tetracaine are applied to the pyriform sinuses with Jackson forceps. The vocal cords are then visualized with a mirror, the blunt tip of a malleable tracheal cannula

*Available from Warren E. Collins, Inc., Boston, Mass., and from McKesson Appliance Co., Toledo, Ohio.

†Available from A. B. Stille-Werner, Stockholm, Sweden.

‡Available from American Cystoscope Makers, Inc., New York, N. Y.

is held just above the cords, and 3 ml. of the 0.25 per cent solution are injected. Contact with the cords should be avoided at this time. The proper placement of the solution is confirmed by explosive cough. A few minutes are again allowed in anticipation of untoward reactions. During this time the fluoroscope, the pressure-flow characteristics of the tube,[39] and the inflatable cuffs on the catheters are checked. If the Carlens catheter is used, the carinal hook is tied to the catheter with silk. The cannula is then placed between the cords and 3 ml. of the dilute tetracaine solution is again injected. This is repeated twice more while the nurse or assistant leans the patient to the left side for adequate anesthesia of the left main bronchus. The last injection should no longer elicit involuntary cough. The patient is encouraged to cough after every injection.

Alternative Anesthesia.—If difficulty is encountered in visualizing the cords in feeble or uncooperative patients, it is at times helpful to apply topical anesthesia by the transcricoid route. Although this method is faster and requires less experience, we have felt that it is more traumatic and that its use is not indicated in patients with tuberculosis.

If prolonged procedures are planned,[37, 40] Forrestier's solution is more satisfactory than dilute tetracaine.

Finally, if the patient is entirely uncooperative, psychotic, or very young, it may be necessary to perform bronchospirometry under general anesthesia. Inhalation anesthesia is preferable since intravenous thiopental often causes laryngospasm and does not obliterate the cough reflex entirely. The addition of curare is undesirable unless facilities exist for attaching the two orifices of the bronchospirometric tube to an anesthesia machine.[36] We have had to resort to general anesthesia on three occasions (Table III).

Intubation.—Judicious haste is now in order. The large size of the catheter is best explained by pointing out that the larger the catheter the easier the breathing. In anticipation of the most frequent questions, patients are told that they will not be able to feel the air move in and out of the lungs, that they may swallow at any time, and that, while the tube is in place, they may breathe through mouth or nose, as desired. A pencil and note pad are supplied so that further complaints or questions may be recorded. The catheter is grasped with the modified Jackson forceps about 15 cm. distal to the tip and is introduced while the epiglottis, cords, and catheter tip are constantly observed in the mirror. Blind introduction usually fails because the tip almost invariably points toward one of the pyriform sinuses. If visualization is poor, it is sometimes helpful to pass the index finger of the left hand along the back of the pharynx, guiding the tube along the midline like a Mosher Life Saver until it passes by the cords. Meanwhile an assistant holds a bite block between the patient's teeth. Proper introduction of the tube should be seen, but can be verified by feeling the air move in and out of the external orifices of the tube and by the patient's inability to phonate.

Alternative Methods of Intubation.—If the tube has not been passed at the first or second attempt, it is best to postpone the procedure. Successive attempts cause more secretions and muscular spasm, and intubation becomes more and more difficult. Occasionally, if a large catheter cannot be passed, a smaller, or female size may be tried. If the catheter tends to buckle, a malleable stylet, introduced through the left lumen of the tube, lends rigidity. Indeed, this is necessary with the Norris tube which does not have a built-in curvature.[3] Occasionally it is easier to pass a straight stylet through the tube and to introduce it by direct laryngoscopy.

Placement of the Catheter.—Improper placement of the bronchospirometric catheter is the most important cause of faulty recording of differential function.

After the catheter has been introduced a soft bite block is placed between the teeth and the patient is lowered to the supine position, the head resting on a small pillow. The catheter is advanced under fluoroscopic observation until the tip has reached the level of the carina. The tip is turned to the left and should be seen to snap over the carina. This identification of the carina is the key to proper placement of the tube.[13] The catheter is slowly advanced until it lies within the bronchus for a distance of two to four centimeters. The location of the tip in relation to the thorax depends on the extent of tracheobronchial distortion (Fig. 4). In patients with contraction of the upper lobe of the right lung, the tip

may appear to lie in the right chest (Fig. 4, *A*). Under these circumstances, advancing the tube until it appears to be properly located in the left chest places the tip in the left lower lobe bronchus with occlusion of the orifice to the upper lobe (Fig. 4, *B*). With deviation of the mediastinum to the left, the properly placed tip may be seen several centimeters to the left of the spine (Fig. 4, *C*). In general, if the tip appears beyond the left border of the heart, the tube has been advanced too far. The appearance of spot films of various tubes in proper position are shown in Fig. 5.

After proper placement the balloons are inflated slowly. The required pressure depends on the thickness of the rubber of the cuffs. It can be determined by inserting the catheter in a transparent tube with an internal diameter equal to that of the trachea or the left main bronchus. Usually 40 to 80 mm. Hg is sufficient. The respiratory orifices of the catheter are

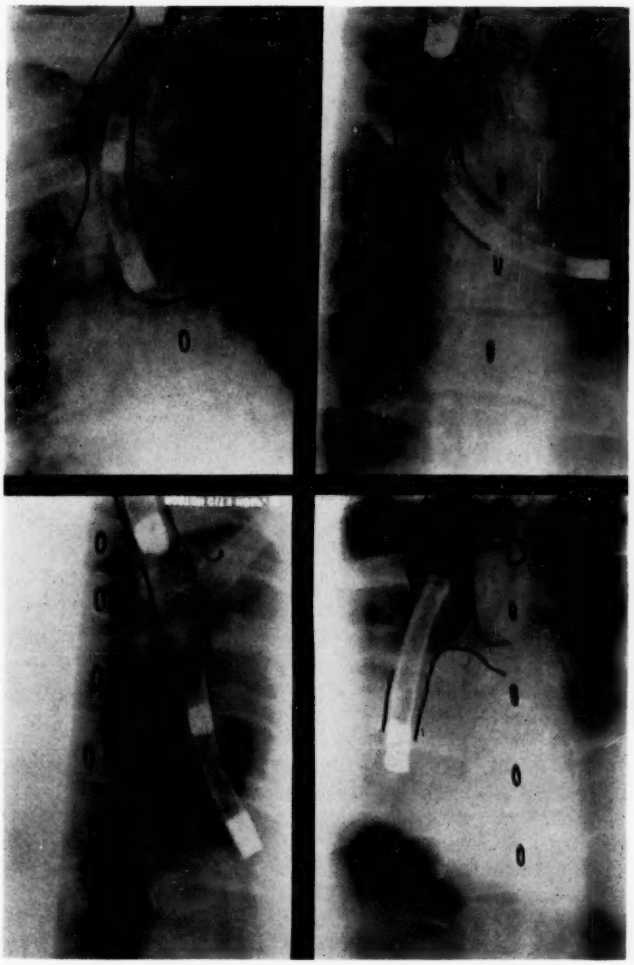

Fig. 4.—Proper orientation is difficult if the bronchial tree is distorted. The first spot film (*A*) shows a correctly positioned catheter in a patient with right tracheal deviation. The tip of the tube appears well to the right of the spine although it lies within the left main bronchus. The second film (*B*) shows the tip of the catheter in its normal relation to the spine. Since there was again marked deviation of the trachea to the right, the tube has been advanced much too far. If the trachea is deviated to the left the tip appears further to the left of the spine than usual (*C*). If the tip cannot be introduced into the left main bronchus because of tracheal distortion or bronchostenosis (*D*) some information can be obtained by advancing the catheter into the right main bronchus.

then connected to the bronchospirometer. We have always used red markers on the the tubes leading to the right lung and the tracheal balloon and red ink for the spirometer pen recording excursions of the right lung. To exclude any possible error the spirometer pens are arranged so that the spirogram of the right lung appears above that of the left (Figs. 3, 6, 10-12).

The Carlens Catheter.—The ligature approximating the carinal hook to the catheter is removed when the rubber hook lies within the trachea. Since this catheter is quite flexible, care must be taken that the gentle tug on the long end of the ligature does not pull the tube out of the trachea. Proper placement is achieved by advancing the catheter until firm resistance is met. Since the catheter is only slightly radiopaque, it is difficult to check proper placement by fluorsocopy (Fig. 5). The addition of radiopaque rings proximal and distal to the bronchial balloon aids in proper fluoroscopic localization. In large patients the rubber hook occasionally fails to engage the carina and passes into the left bronchus. If the tube is used in the operating room the position of the catheter should be checked by spot films or tracings before operation.[36]

Difficulty with Placement.—This is almost always due to deviation of the trachea to the right or to narrowing of the left main bronchus.

If the trachea is shifted to the right, the left bronchus may take off at a 90 degree or sharper angle (Fig. 4, *A, D*). The tip then tends to slide into the right bronchus (Fig. 4, *D*). Under these circumstances placement is facilitated by bending the patient's head sharply to the right until the right ear approaches the right shoulder.

When the left main bronchus is narrowed the catheter points in the proper direction but fails to advance. If the obstruction is known to be due to fibrosis it is permissible to apply slight pressure accompanied by to and fro rotation of the tube. If the cause of the stenosis is unknown, or due to tumor or inflammatory lesion, this maneuver is not permissible. If this fails a smaller tube may be tried, preferably on another day, or the catheter may be advanced two to three centimeters into the *right* bronchus (Fig. 4, *D*). Inflation of the bronchial balloon then obstructs the right upper-lobe bronchus. The upper spirogram, which ordinarily depicts excursions of the right lung, now represents excursions of the left lung. By comparing this tracing with the ordinary spirogram, some idea can be formed concerning the functional participation of the left lung.

Recording the Bronchospirogram.—Although it has been suggested that the procedure should be carried out on two separate occasions to assure correct results,[35] we feel that this is seldom necessary. The following outline may prove helpful in removing doubts concerning the reliability of results. After positioning of the catheter and balloon inflation, a spot film of the trachea is made and recording is begun. If the tracing appears satisfactory, recording is continued for at least four minutes. Up to ten minutes may be required if breathing is irregular or if the pulmonary midposition changes frequently. A second spot film may then be made to assure that the position of the catheter has not changed. The tip of the catheter is again visualized fluoroscopically. If it appears close to the carina, the tube is advanced about one centimeter. If it appears near or beyond the left border of the heart, the catheter is withdrawn about one centimeter. A third spot film is made and a second tracing is recorded for four to ten minutes. The slight alteration in position of the catheter between the duplicate recordings assures that in at least one tracing the orifice to the left upper lobe was not blocked. A final spot film is exposed before removing the catheter. If no technical difficulty was encountered, if comparison of the first with the second and the third with the fourth spot films shows no alteration in position of the tube during recording, if the spot films show the catheter not less than 2 cm. and no further than 4 cm. within the left main bronchus in either set of films, and if the two bronchospirograms show identical functional distribution, results can be reported with confidence. This outline was followed in most of our examinations.

Detection of incorrect position and of technical difficulties depends to a great extent on correct interpretation of the tracing.

INTERPRETATION

The Normal Bronchospirogram and Derived Data.—The results by others, concerned with normal functional division between the two lungs, have been summarized.[31]

In eighteen normal volunteers examined by us the right lung was responsible for 51 to 62 per cent of the total function. In any given individual the distribution of oxygen uptake, ventilation, and vital capacity was usually the same. Three representative bronchospirograms of normal male volunteers are reproduced in Fig. 6. In these and all subsequent reproductions the ventilation of the right lung is recorded above that of the left, and the spirogram reads from right to left. The heavy horizontal rulings represent a volume of 500 ml.,

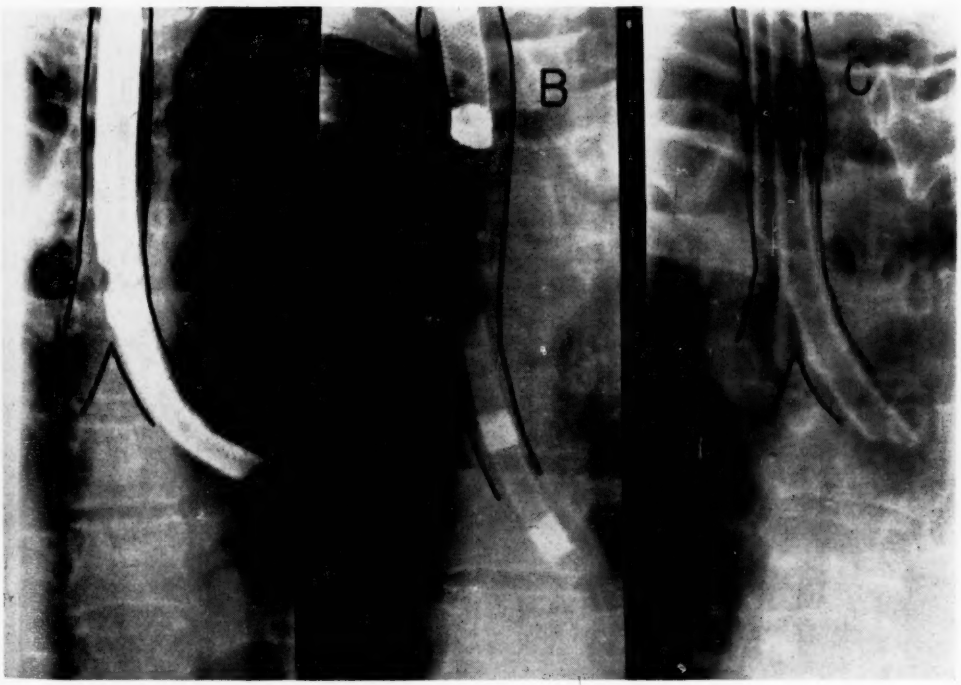

Fig. 5.—Spot films of correctly placed Zavod (*A*), Gebauer (*B*), and Carlens (*C*) catheters. The rubber hook of the Carlens catheter can be seen to engage the carina.

and the heavy perpendicular rulings a time interval of one minute. Although the first two patients breathed more regularly than the third, all three tracings could be adequately interpreted. The total vital capacity was well over 3,000 ml. in each instance.

A convenient form for calculating and reporting the results is shown in Fig. 7. The respiratory rate is counted for a minute interval. The mean tidal volumes of the two lungs can be estimated by drawing two lines, one for the end-expiratory points and one for the end-inspiratory points. A more accurate estimate of the mean tidal volumes can be obtained by measuring each tidal stroke and adding all excursions for one minute. The mean tidal volume is

obtained by dividing the minute ventilation by the respiratory rate. Ventilographs permit direct reading of the minute ventilations.[37] The newly proposed nomenclature is used for the subdivisions of the vital capacity.[41] The two inspiratory capacities and the two expiratory reserve volumes are measured, and the vital capacities are obtained by addition of these two volumes. The oxygen uptake is represented by the slope of the base line as for the determination of the basal metabolic rate. The ventilation equivalent for oxygen is defined as the number of liters of ventilation necessary for an oxygen uptake of 100 ml. This expression is of greater importance in bronchospirometry than in ordinary spirometry because, in the absence of arterial hypoxia, the two ventilatory equivalents give an indication of the relative ventilation-perfusion relationship in the two lungs. The numerical data read from the tracings are absolute values and have little significance. The functional distributions, expressed as per cent of the total, are of importance and are entered in the form in the area blocked out by heavy lines (Fig. 7).

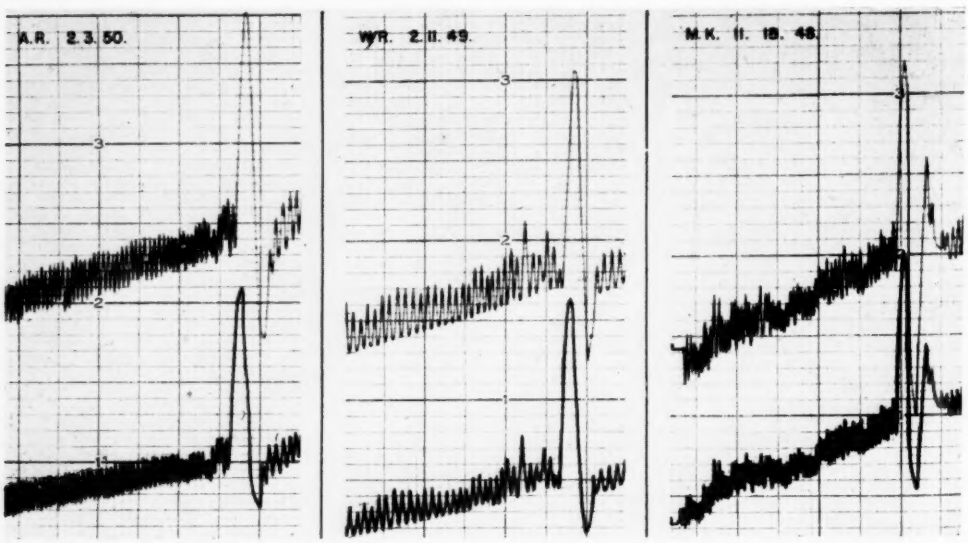

Fig. 6.—Bronchospirograms of three normal male volunteers. In every instance the right lung was responsible for a somewhat larger oxygen uptake, ventilation, and vital capacity. The first tracing (A. R.) was entirely satisfactory, the second tracing (W. R.) was only moderately satisfactory because there were considerable variations of tidal excursions. The third tracing (M. K.) was only fair since respiratory rate, tidal excursions, and functional residual capacity all varied markedly from one moment to the next.

Faulty Bronchospirograms.—1. Improper placement of the catheter: The commonest cause of failure to record actual functional distribution is improper placement of the tube. The first tracing (*A-1*) of Fig. 8 illustrates the result when the catheter is advanced too far so that the bronchial cuff occludes the left upper-lobe bronchus. Slight withdrawal of the catheter permitted ventilation of the left upper lobe. The second tracing (*A-2*) and the corresponding two spot films (Fig. 9, *A-1* and *A-2*) made the initial error evident.

A more unusual misplacement is illustrated by the second set of spirograms in Fig. 8. Collapse procedures applied to the apex of the left lung may force

the upper lobe bronchus downward so that the catheter may enter it. A tracing (*B-1*) was first obtained under such circumstances in a patient who had had an extensive apicolysis followed by extrapleural pneumothorax. The lower spirogram shows the respiratory excursions of a nearly functionless left upper lobe.

Physiology Laboratory, Sanatorium Division, Boston City Hospital

DIFFERENTIAL BRONCHOSPIROMETRY

Name _____ Sex ___ Age ___ Date _____

Weight _____lbs. Height _____ft. Surf.Area____m^2

	Tracing #1			Tracing #2		
	Right	Left	Total	Right	Left	Total
Respir.Rate						
Tidal Volume						
Inspiratory Cap.						
Expiratory Res.						
Vital Capacity						
Minute Volume						
Min.Oxygen Upt.						
Ventilatory Equ.						
Oxygen Uptake %						
Ventilation %						
Vital Capacity %						
Inspiratory Cap.%						
Expiratory Res. %						

Type of Tracing _____ _____

Position of Catheter _____ _____

Remarks _____

Postop. Course _____

Fig. 7.—A form for recording, calculating and reporting results of bronchospirometry. Only the values recorded within the heavily ruled block are considered significant.

The tracing (*B-2*) was next obtained after the catheter had been slightly withdrawn and reintroduced. The spot film revealed the tip now within the lower-lobe bronchus and the orifice to the upper lobe was occluded much as in tracing *B-1*. At last the catheter was withdrawn about 2 cm. and the third tracing (*B-3*) showed the true functional distribution. Such different and bizarre results can only be evaluated later by comparison of repeated tracings and corresponding well-marked spot film. The next two tracings (*C*) illustrate a type of misplacement which may be easily overlooked. In the early part of the tracing

(*C-1*) the left lung appears to be absorbing more oxygen, although the right lung ventilates more. This paradoxical situation has been encountered quite frequently.[42] The left lung, however, had a much smaller vital capacity than the right. This is unusual, as oxygen uptake and vital capacity are usually proportionately reduced. The tube was advanced about 1 cm., and a second tracing was obtained (*C-2*). The actual relationship of functional participation between the two lungs was now evident. Later inspection of the spot films (Fig. 9, *C-1* and *C-2*) revealed that the tip of the catheter had been in the trachea during the first tracing (*C-1*). The resistance of the two airways was

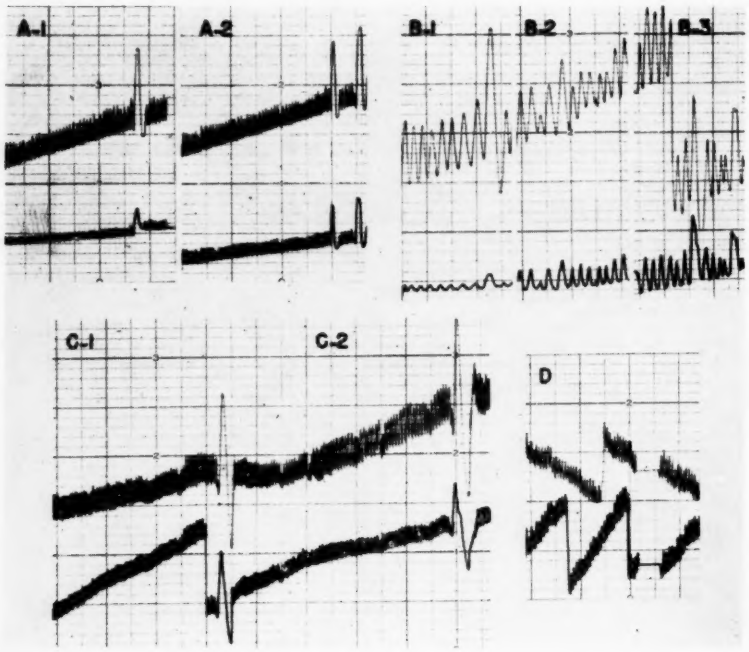

Fig. 8.—Faulty bronchospirograms due to improper placement of the catheter. The tube was advanced too far with resulting occlusion of the right upper lobe bronchus (*A-1*). Slight withdrawal corrected this malplacement (*A-2*). In a patient with a depressed left upper-lobe bronchus the tube may actually enter this bronchus, occluding the orifice to the left lower lobe (*B-1*). Withdrawal and readvancement resulted in occlusion of the left upper lobe bronchus (*B-2*). The bronchospirogram with the catheter in the proper position is shown last (*B-3*). The tip of the catheter was within the trachea in the next tracing (*C-1*). Slight advancement of the catheter corrected this mistake (*C-2*). The last tracing (*D*) shows the more common appearance if both orifices of the catheter remain within the trachea.

such that, although both distal orifices of the catheter had been within a common chamber in the trachea, a fairly plausible, though entirely misleading bronchospirogram had been obtained. The last tracing (*D*) shows the more usual appearance when the tip of the catheter remains within the trachea. The only difference between tracing *D* and tracing *C-1* is that the air leak from one side to the other was slower in the latter.

2. *Technical difficulties:* The tracings resulting from some common technical difficulties are reproduced in Fig. 10. In general, if variations of the baselines of the two spirograms occur simultaneously and in the same direction the

change is due to an alteration in the pulmonary midposition (functional residual capacity). This may be voluntary (*A*), it may occur immediately after a vital capacity effort (*B*), or it may be due to a sudden alteration in oxygen requirement (*C*). On the other hand, if the base lines of the two tracings move in opposite directions, a leak almost certainly exists. This may occur at any level of the separate airways. The fourth tracing (*D*) of Fig. 10 initially shows the last portion of a satisfactory spirogram. The balloons were then deflated,

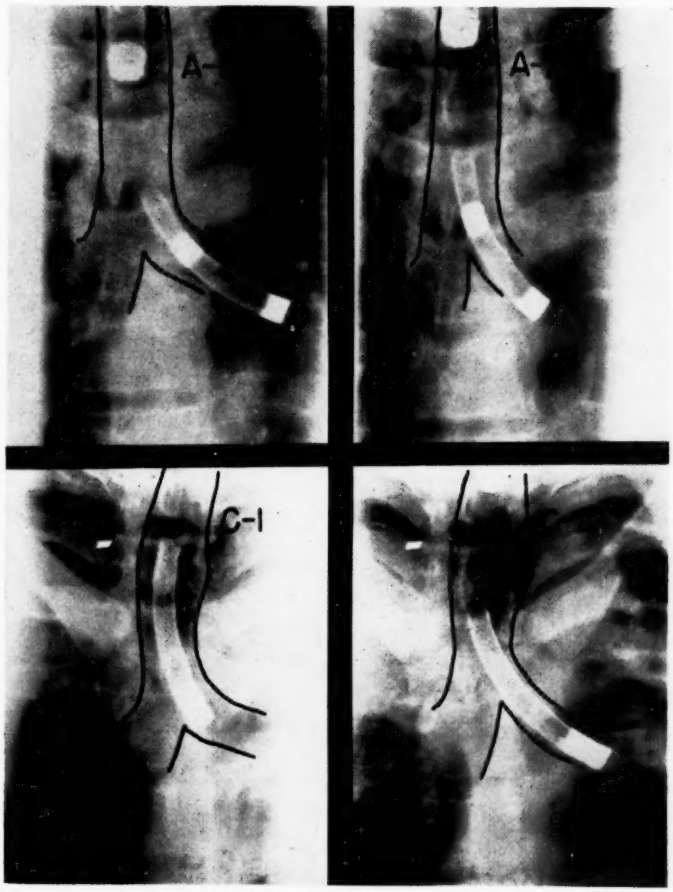

Fig. 9.—Spot films of the Gebauer catheter in situ. The first film (*A-1*) shows the tip of the catheter advanced too far with resulting occlusion of the left upper bronchus. The second film (*A-2*) shows the correct position. The two resulting tracings are shown in Fig. 8, *A-1* and *A-2*. The third film (*C-1*) shows the catheter not advanced far enough. Although the tip is within the left main bronchus, the bronchial balloon is still within the trachea. The last film (*C-2*) shows the corrected position. These two positions resulted in tracings *C-1* and *C-2* of Fig. 8.

the catheter withdrawn slightly, and the second recording begun at the first arrow. The bronchial balloon was reinflated too forcefully and the catheter tip slipped into the trachea. A leak from one lung to the other caused a sudden alteration in the relationship of tidal volumes. Slight advancement of the catheter and gentle reinflation resulted in resumption of acceptable recording at the second

arrow. The next tracing (*E*) shows a marked leak from left to right which could not be remedied by balloon reinflation or change in catheter position. The catheter was removed, and later examination revealed that a stylet had torn the dividing wall between the two lumina of the tube resulting in an air leak within the catheter. The next tracing (*F*) demonstrates that a leak may exist even though both spirograms proceed in an upward direction. At first

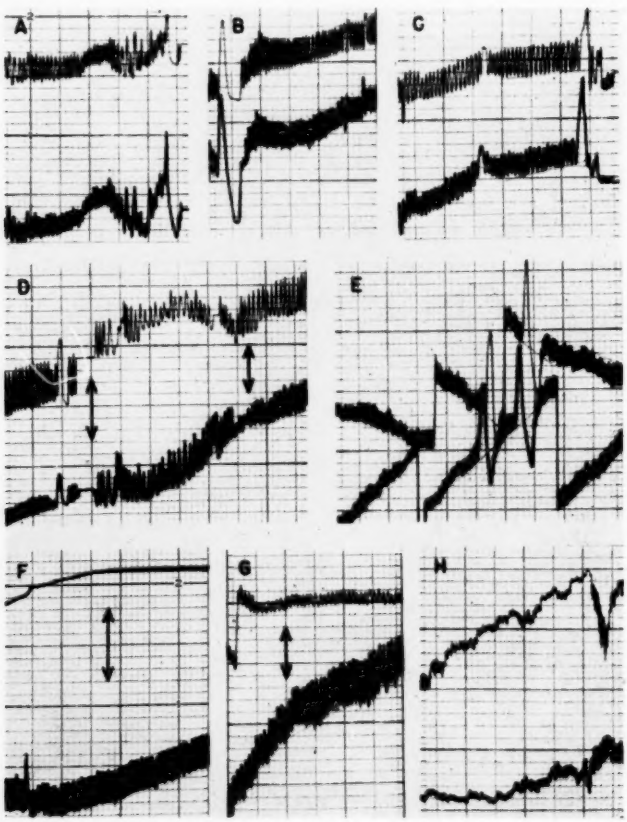

Fig. 10.—Faulty bronchospirograms due to technical difficulties. The first three tracings (*A*, *B*, and *C*) are in order, since the marked variations of the base line occur in parallel fashion. Rapid inflation of the bronchial balloon forced the tip of the catheter back into the trachea during the time interval marked by the two arrows (*D*). A leak (*E*) was due to a defective wall between the two lumina inside the catheter. Insufficient balloon inflation, corrected at the arrow, caused a leak in the next spirogram (*F*) and a similar defect was due to abnormal communication between the twin spirometers in the next tracing (*G*). Cough and excessive secretions caused intermittent leak and changes in catheter position in the last tracing (*H*).

the pressure within the balloons was insufficient to prevent a slow air leak from the right to the left side, thus simulating oxygen uptake on the right. Greater inflation of the balloons (arrow) interrupted this leak, and the right lung was shown to be functionless. A flow of air from the left to the right side was initially observed in the next bronchospirogram (*G*) in spite of proper balloon inflation and proper positioning of a well-tested tube. Inspection of the entire apparatus revealed that both oxygen supply cocks had been left open and, al-

though the tank was tightly closed, air moved from one spirometer to the other via this common tubing. After closing the cocks (arrow) a satisfactory bronchospirogram was recorded. The result of intermittent leaks and obstruction due to secretions is illustrated in the last tracing (*H*).

3. *Unilateral airway obstruction:* A common difficulty encountered with catheters whose airway for the right lung ends in a lateral orifice[31, 39] is obstruction of the opening by a deformed trachea (Table IV). This is shown by the first three tracings reproduced in Fig. 11. The first patient (*A*) had a left pneumothorax. In spite of this the expiratory excursions were smaller on the

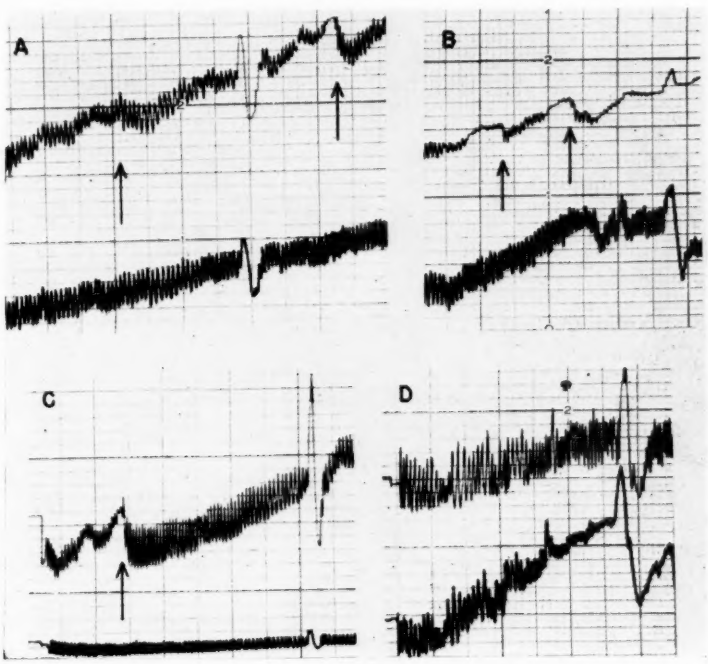

Fig. 11.—Faulty bronchospirograms due to unilateral airway obstruction. This is usually due to occlusion of the lateral orifice of the right lumen of the catheter by a deviated trachea (*A*, *B*, and *C*). The obstruction may be partially (*A* and *B*) or completely (*C*) corrected by flexing the patient's head sharply to the right. Increasing obstruction of the left airway (*D*) was due to excessive accumulation of purulent material.

right. At a point indicated by the arrow, the patient's head was bent to the right altering the curvature of the trachea in the area of the lateral tube opening. Relief of the obstruction can be observed from the upper tracing. An elevated pulmonary midposition (functional residual capacity) due to "air trapping" was reduced, the base line descended, and the tidal volume increased. When the head was again straightened the respiratory excursions became progressively smaller and a sharp rise of the base line indicated renewed "trapping" of air. A second bending of the head (second arrow) brought even more evident relief. A similar obstruction in a patient with a small right thoracoplasty was so severe that bending of the head to the right shoulder brought only partial relief, and a satisfactory spirogram could not be obtained with this

catheter (*B*). The best illustration of unilateral obstruction and its complete relief by bending the head for the remainder of the test is shown in the third bronchospirogram (*C*). This difficulty cannot occur with the use of tubes where both airways have end-openings.[31] Progressively increasing obstruction of the left airway due to accumulated secretions is illustrated in the last tracing (*D*). A final attempt by the patient to relieve the obstruction by cough merely expelled some of the "trapped" air from the left lung.

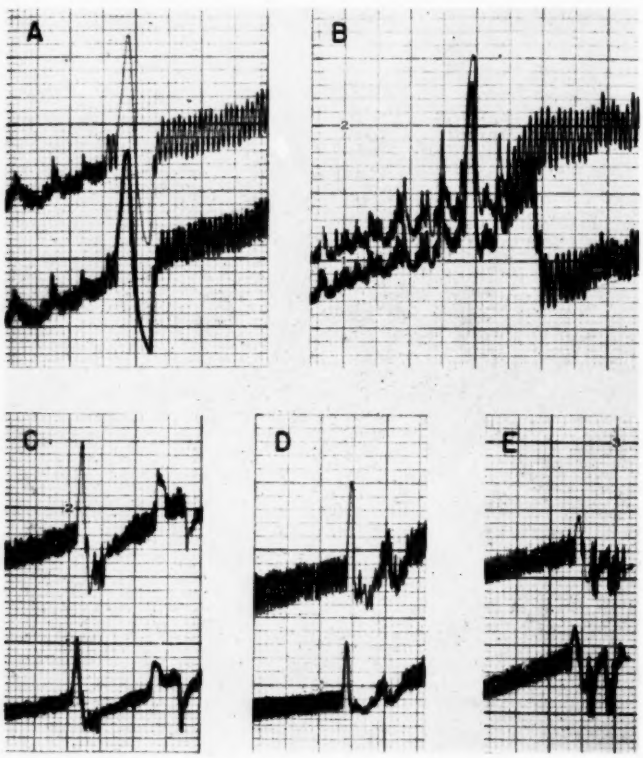

Fig. 12.—Irregular, rapid, shallow respirations are often due to fear of an inadequate airway. This is usually corrected by an early vital capacity effort (*A* and *B*). If the tracing is satisfactory the vital capacity effort is best deferred until last since the forced expiratory effort occasionally mobilizes secretions and the procedure is prematurely terminated by uncontrollable cough (*C*, *D*, and *E*).

Timing of the vital capacity effort: A satisfactory tracing often depends on proper timing of the vital capacity effort (Fig. 12). The patient's fear of an inadequate airway is usually reflected by irregular, rapid, shallow respirations. A vital capacity effort shows him that large volumes of air can be exchanged with relative ease and respirations become more regular (Fig. 12, *A*, and *B*). If the tracing is satisfactory the vital capacity effort is best deferred until a tracing of sufficient duration has been obtained since the forced expiratory effort occasionally causes mobilization of secretions, uncontrollable cough, and premature termination of the procedure (Fig. 12, *C*, *D*, and *E*).

POSTOPERATIVE CARE

Removal of the catheter after 0.25 per cent tetracaine anesthesia is always accompanied by spontaneous and effective cough. The patient is then placed in the prone position and the table is tilted down until he is able to touch the floor with his hands. After this postural drainage he is returned to the ward. Nothing is given by mouth for two hours to avoid aspiration. Parenteral penicillin is given twice a day for three days or until the temperature has been normal for three days. A four-hour temperature chart is kept during this period. The patient should be aroused frequently during the afternoon after the procedure, and postural drainage is continued at frequent intervals for three days. Narcotics, hypnotics, or sedatives are not allowed in patients with pulmonary tuberculosis or with copious secretions.

Since sedation has been eliminated both pre- and postoperatively, fewer bronchospirograms have shown perfectly even and regular respiratory excursions. The procedure also appears somewhat more formidable to patients who have not been sedated, and the salivation without use of atropine makes the conduct of the anesthesia and the recording somewhat more difficult. These disadvantages are far outweighed by the significant reduction in the number of postoperative febrile reactions and exacerbations of the disease in patients with pulmonary tuberculosis.

A quiet and orderly team, well designed and well functioning apparatus, thorough topical anesthesia, careful placement of the catheter and correct interpretation of the tracings are the essentials of satisfactory bronchospirometry.

SUMMARY

Complications of bronchospirometry were few in number.

Among 107 patients who received topical anesthesia with Forrestier's solution there were no toxic reactions. Forty per cent of patients with bronchial asthma showed an allergic response. Among 753 patients who received 2 per cent tetracaine pharyngeally and 0.25 per cent tetracaine intratracheally, 0.3 per cent had a mild toxic reaction and 44 per cent of asthmatics showed an allergic response in the form of an acute attack.

Of 97 patients with tuberculosis who received a barbiturate, atropine, morphine and Forrestier's solution preoperatively, 48 per cent had a febrile reaction, and 8.3 per cent showed an extension of disease by roentgenogram. Of 333 patients with tuberculosis who received only a barbiturate followed by dilute tetracaine anesthesia, 21 per cent showed a febrile reaction and 0.3 per cent showed a spread of disease.

Both febrile reactions and spreads were more common in patients with far-advanced unstable disease and in toxic patients with severe symptoms.

Ulcerative tracheobronchial tuberculosis and hemoptysis within two weeks were accepted as absolute contraindications to bronchospirometry. Obstruction of the left main bronchus and far-advanced pulmonary tuberculosis with severe symptoms were relative contraindications. Dyspnea was not considered a contraindication, and the examination was performed without mishap in patients with all degrees of pulmonary insufficiency.

The description of technique includes preliminary studies, preoperative treatment, equipment and technical preparation, application of topical anesthesia, intubation, placement of the catheter, and recording of the bronchospirogram. The importance of obtaining duplicate tracings and of checking catheter position by spot films is emphasized.

Normal bronchospirograms are shown, and the calculation of derived data is discussed. The appearance of the bronchospirograms with improper placement of the catheter, with various technical difficulties and with unilateral airway obstruction is illustrated.

Early re-establishment of the cough reflex and effective removal of secretions were facilitated by avoiding postoperative sedation and by intensive postural drainage.

Adequate information concerning the differential functional participation of the two lungs was obtained in 94 per cent of all procedures.

The authors acknowledge the nursing and technical assistance of Miss H. Bruzzo, Miss D. M. Donnelly, Miss M. Jacobs, Miss K. Paipulas, and Miss S. S. Smith.

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A COMPLETE SOMATIC ANTIGEN COMMON TO SALMONELLA ADELAIDE, ESCHERICHIA COLI-GOMEZ, AND ESCHERICHIA COLI O111:B4

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México, D. F.

VARELA and associates (1946) isolated a bacterium from the feces and purulent secretion from the external ear of a 2-month-old child who died of acute enteritis. The microorganism was classified as a member of the Escherichia group. The bacterium was peculiar in that it possessed the biochemical characteristics of *Escherichia coli* and the complete somatic antigen of *Salmonella adelaide* (XXXV, of the Kauffmann-White schema). These investigators also reported the isolation of this *Escherichia coli* serotype during an outbreak of diarrhea that involved five children in the Hospital Infantil of Mexico City, and from a woman who worked in the kitchen of the hospital. Microorganisms of this type were named *Escherichia coli-gomez*.

At first, *E. coli-gomez* cultures were tested with Salmonella agglutinating antisera in an effort to find an antigenic fraction which might indicate pathogenicity or which might serve to identify them. Later, when the Kauffmann-Knipschildt-Vahlne schema was established for the Escherichia group, we found that *E. coli-gomez* belonged to Escherichia O group 111. The results of our studies on the antigenic relationship of *E. coli-gomez*, *E. coli* O111:B4, and *S. adelaide* are given below.

The methods used for the preparation of antigen suspensions and antisera, for performance of agglutination tests, and for absorption of agglutinin were those advised by Kauffmann in 1951 (*a*). Cultures of *E. coli-gomez* were available locally and the *E. coli* O111:B4 (Stoke W) and *S. adelaide* cultures were received from Dr. Edwards and Dr. Ewing of the Communicable Disease Center, Atlanta, Ga. Antisera for the Stoke W strain of *E. coli* O111:B4 and for *E. coli-gomez* were prepared by the injection of living cultures. The antiserum for *S. adelaide* was prepared from a culture that was heated at 100° C. for one hour.

Heated suspensions of *E. coli-gomez*, *E. coli* O111:B4, and *S. adelaide* cross agglutinated to high titer in the unabsorbed antiserum prepared with these three cultures (Table I). Also the results of reciprocal agglutinin absorption tests between *E. coli-gomez* and *E. coli* O111:B4 indicate that these two serotypes have identical somatic antigens since all agglutinin was removed from the antisera. Further, it was shown by reciprocal agglutinin absorption tests that the somatic antigens of *S. adelaide* and these *Escherichia* cultures are identical.

These results were communicated to Dr. Kauffmann who confirmed the relationships outlined herein (Kauffmann, 1951 [*b*]). Therefore, *E. coli-gomez*

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TABLE I. CROSS AGGLUTINATION REACTIONS WITH HEATED CULTURES

ANTIGEN SUSPENSIONS	ANTISERUMS								
	E. COLI O111:B4			E. COLI-GOMEZ			S. ADELAIDE		
	UNABSORBED	ABSORBED BY E. COLI-GOMEZ	ABSORBED BY S. ADELAIDE	UNABSORBED	ABSORBED BY O111:B4	ABSORBED BY S. ADELAIDE	UNABSORBED	ABSORBED BY E. COLI-GOMEZ	ABSORBED BY O111:B4
E. coli O111	3200	0	0	6400	0	0	1600	0	0
E. coli-gomez	3200	0	0	6400	20	20	1600	0	0
S. adelaide	800	0	0	3200	0	0	3200	0	0

(Varela and associates, 1946) corresponds to *Bacterium coli neopolitanum* described by Bray and Beavan (1948), to *Bacterium coli* α type of Giles and Sangster (1948), and to *E. coli* O111:B4, Kauffmann and Dupont, 1950. Somatic antigen XXXV of *S. adelaide* is identical with the somatic antigen of *E. coli* O111. According to Kauffmann (1951 [*b*]) antiserum for *E. coli* O111:B4 is absorbed completely by *S. adelaide*, it being impossible to isolate the B4 factor in pure form. The relationships between *E. coli* O111:B4 and *S. adelaide* also were confirmed by Ewing (1951).

E. coli O111:B4 has been encountered in several outbreaks of infantile gastroenteritis in England, Scotland, United States, the Netherlands, Sweden, and Denmark, showing an evident causal relationship to the disease. Although numerous coliform organisms have Salmonella antigens, it is interesting to note this major relationship between *E. coli-gomez*, *E. coli* O111:B4 and the Salmonella XXXV antigen. Frantzen (1951) has also described a similar relationship between microorganisms of the Arizona group and *E. coli* O55:B5. The latter serotype also has been implicated as a cause of infantile diarrhea.

SUMMARY

E. coli-gomez (Varela and co-workers, 1946) and *E. coli* O111:B4 (Kauffmann and Dupont, 1950) are identical, and at the same time have the Salmonella somatic antigen XXXV. It is interesting that this supposedly pathogenic coliform organism has a major somatic antigen of the Salmonella group.

We gratefully acknowledge indebtedness to Dr. F. Kauffmann, Copenhagen, Denmark, and Dr. W. H. Ewing, Atlanta, Ga., U. S. A.

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THE EFFECT OF PARTICLE SIZE ON BLOOD CLEARANCE AND TISSUE DISTRIBUTION OF RADIOACTIVE GOLD COLLOIDS

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FOR some time it has been desirable to know how the properties of particles influence their deposition in various organs and, if possible, in various cell types. It seems probable that the fate of colloidal particles is determined by surface forces resulting from the chemical nature of the particle and its size.

Dobson and associates[1] have shown a marked effect of the particle size of radioactive yttrium and zirconium colloids on the site of their deposition and blood clearance rate in rabbits. Sheppard and associates[2] found no significant difference between the blood clearance rates of large and small particles of radioactive gold in dogs. The data obtained by these two groups of investigators cannot easily be compared because of the differences in the chemical nature of the colloids and in the animal species employed, and because of the lack of data on the particle sizes employed.

In this paper, we report a study of the removal of different sized radioactive gold colloids from the blood of dogs and their distribution in various tissues. Radioactive gold was chosen because of availability, ease of measurement, and because much is known about its chemical preparation to particles of approximately known size.

EXPERIMENTAL

Radioactive gold sols were prepared and their particle size measured as described in a previous communication.[3] The absence of ionic gold in the sol was tested with o-tolidine,[4] and was confirmed by the fact that no radioactivity could be detected in the urine of dogs as long as two hours after intravenous administration of the colloid.

A portion of the colloid containing between 1 and 2 mc. of Au^{198} was prepared for injection by mixing it in vitro with 4 to 5 ml. of the dog's own serum. After standing for a few minutes, the solution was then injected intravenously into the dog, which was under Nembutal anesthesia. Blood samples were taken in a heparinized syringe from 5 to 120 minutes after the injection. Aliquots were diluted with water, and 10 ml. portions were counted for 5,000 counts in a flat dish under an end-window Geiger tube. After all the blood samples were taken, tissues were removed from some of the dogs for analysis. They were weighed and heated with nitric acid until all particulate matter had dissolved. At the end of the digestion, at least 2 ml. of concentrated HCl was added to transform the metal into ionic gold. This greatly reduced the adsorption of radioactivity on the walls of the glass vessels. Because of their importance, the entire liver and spleen were analyzed. After the digestion of these large

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organs, an appreciable amount of fatty material appears on top of the water phase which made taking of an aliquot portion and counting of liquid samples rather inaccurate. To obviate this difficulty, the digest, after cooling to room temperature, was mixed with acetone or dioxane until the fatty layer and water phase had been transformed into a one-phase system.[5] Ten milliliter portions were counted as before. Ninety-five to one hundred per cent of Au^{198} added in vitro to nonradioactive fat-containing organs could thus be recovered.

RESULTS

In Fig. 1 some typical blood clearance curves are presented as a semilogarithmic plot of Au^{198} concentration against time. The curves on top are

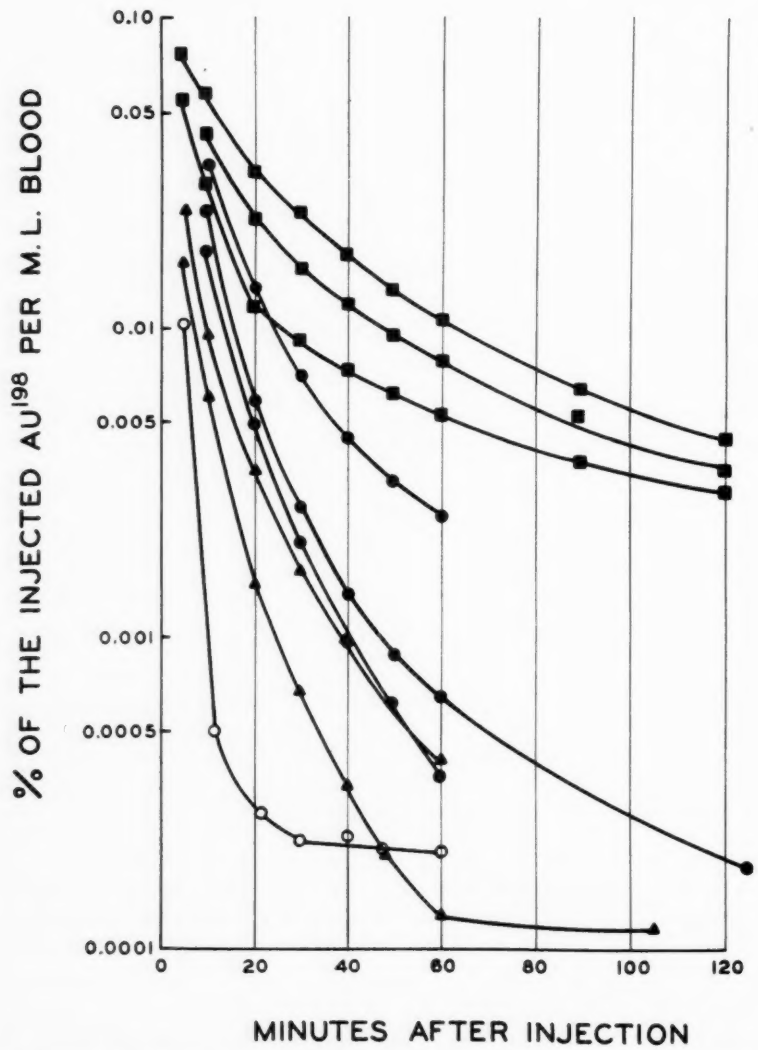

Fig. 1.—A semilogarithmic plot of the disappearance of gold colloids from the blood stream. ■ 10 mμ diameter; ● 20 mμ diameter; ▲ 27 mμ diameter; ○ 40 mμ diameter.

those for gold particles of approximately 12 mμ in diameter, whereas the lowest curve represents 40 mμ particles. The others represent the disappearance of intermediate-sized particles.

Even though some of the curves can be accurately fitted by the equation $C = a_1e^{-b_1t} + a_2e^{-b_2t}$, others seem to require more terms. An accurate analysis of the curves was not practical because of the short duration of the experiment. Therefore, in Table I the decrease in concentration of radioactive gold in blood is listed. The time after injection when the concentration of Au^{198} in the blood reaches one-half of the extrapolated zero time value is designated as $t_{1/2}$; $t_{1/10}$ is the time when the concentration has decreased to one-tenth of the zero time value. Even though there is a wide spread of values within each category of particle size, there can be no doubt that there is a real increase in the rate of disappearance of gold particles as their size increases. Since the blood clearance rate might depend upon the number of particles injected, dogs 1, 2, and 3 were injected with the same number of 10 mμ particles as were given to dogs 15 and 16, in the form of 47 mμ particles. Moreover, two dogs (No. 4 and No. 5) were each injected with the same number of particles, of the above sizes successively. In both instances the large particles disappeared more rapidly.

TABLE I. BLOOD CLEARANCE TIME OF DIFFERENT SIZED GOLD PARTICLES

DOG NO.	WEIGHT (KG.)	SEX	PARTICLE DIAMETER (mμ)	CLEARANCE TIME $t_{1/2}$ (MIN.)		CLEARANCE TIME $t_{1/10}$ (MIN.)	
1	8.0	M	10	11.5		45	
2	10	M	10	5.3		18	
3	11.5	M	10	6.3		20	
4	13.8	F	10	6.5	Av.	27	Av.
5	11.8	M	10	7.0	8.2	22	34
6	9.3	M	12	13.0		63	
7	10.5	M	12	9.1		48	
8	8.4	M	12	6.3		26	
9	8.2	M	20	4.8	Av.	14	Av.
10	10.4	M	20	4.5	5.3	18	19
11	8.7	M	20	6.5		24	
					Av.		Av.
12	17	M	27	2.5	2.8	10	10
13	10.5	M	27	3.0		10	
14	9.0	M	40	1.5		5	
15	10.0	F	47	2.0		10	
16	16.0	F	47	2.8	Av.	8	Av.
4	13.8	F	47	1.5	2.1	5	7
5	11.8	M	47	2.7		8	

It should be noted that nearly all curves upon extrapolation to zero time gave a volume equivalent to 8 to 10 per cent of the body weight. This is close to the blood volume of the dog, which is to be expected since whole blood was counted. This close approximation of the blood volume by extrapolation is evidence that during the first five minutes the rate of disappearance is not much greater than the rate immediately after the five-minute point.

The data obtained on the dog tissues are presented in Table II. Liver and spleen are by far the most active in removing both large and small gold

TABLE II. PERCENTAGE OF THE INJECTED Au^{198} FOUND IN VARIOUS TISSUES

DOG (KG.)	PARTICLE DIAMETER (mμ)	LIVER		SPLEEN		BLOOD		MARROW	LYMPH NODE	MUSCLE	TOTAL†
		PER W.O.*	PER KG.	PER W.O.	PER KG.	PER W.O.	PER KG.	PER KG.	PER KG.	PER KG.	
8.0	10	82	230	2.1	125	2.4	3.0	5.0	1.0	0.00	87
11.5	10	83	210	1.7	43	0.5	0.4	5.0	2.0	0.02	85
7.7	10	--	--	2.0	60	0.2	0.3	--	2.5	0.03	--
8.7	20	87	340	2.1	56	2.2	2.5	2.0	0.7	0.07	91
10.4	20	--	--	1.5	52	0.4	0.4	4.0	1.0	0.02	--
10.5	27	80	250	4.1	55	5.0	5.0	--	--	--	89

*Per W.O.—Per whole organ. For blood this is calculated by multiplying the Au^{198} in the last sample by 10 per cent of the body weight.
†Recovery in liver, spleen, and blood.

particles from blood. Eighty to ninety per cent of the injected dose was recovered in these two organs as has been reported by many investigators. Per gram of tissue, the spleen takes up only one-half to one-fifth as much as the liver. The other phagocytic tissues like bone marrow and lymph nodes are much less active in the removal of gold particles from the blood. In two dogs the uptake of colloidal gold per gram of kidney and lung was found to be of approximately the same magnitude as that of bone marrow. Muscle, a tissue which has no known phagocytic action, did not pick up colloidal gold. The small amount of radioactivity present in that tissue can be easily accounted for by the blood in the sample.

DISCUSSION

The data presented in this paper confirm previous work by Sheppard and associates[2] to the extent that gold colloids are rapidly removed from the blood stream of dogs. In contrast to their studies, we found a definite dependency of the blood clearance rate on the size of the particle injected. In addition, we did not find "bumps" in the disappearance curves. This last difference cannot be explained by the fact that our dogs were under Nembutal anesthesia, whereas theirs were not anesthetized.* We performed experiments on several unanesthetized dogs which did not show irregularities in the disappearance curves. The only other obvious difference between their technique and ours is the preparation of the gold sol previous to injection. They used a sol prepared by the reduction of Au^{+++} by ascorbic acid, whereas we used sols produced with yellow phosphorus and H_2O_2. Moreover, we mixed the gold sol with the dog's own serum in vitro several minutes before injection. This was done in order to prevent the interaction of the gold particles with serum proteins simultaneously with their removal from the blood stream. We hoped that coating of the particles with serum proteins in vitro would make the effect of the rate of injection on the resulting blood clearance curves of less importance.

One of our observations would support the idea that serum protein-coated particles are handled differently from particles coated with other materials. In one dog we injected a serum-coated gold sol, followed after two hours by a gelatin-coated gold sol, which in turn was followed two hours later by an oleic acid-coated gold sol. The disappearance of the last two colloids was more rapid than the first, and in both cases definite bumps were observed twenty to fifty minutes after injection, whereas a smooth curve was observed for the serum-coated colloid. We have measured the blood clearance curves of serum-coated gold particles of different sizes twenty-three times, and have seen only smooth disappearance curves. The problem of the influence of particle envelope on the fate of injected colloids deserves further study.

The shape of the blood disappearance curves of Fig. 1 deserves some comment. If the rate of removal of gold particles had been proportional to the concentration of the particles in the blood, we should have obtained straight lines in a semilogarithmic plot. It is obvious that the curves in Fig. 1 show definitely

*In one of the experiments of Sheppard and associates,[2] a smooth curve was obtained. In all others a marked irregularity occurred ten to thirty minutes after injection of the gold colloid.

slower disappearance rates as time goes on. One might suggest that the reason for the slower rate of disappearance at the later time intervals would be the gradual saturation of the mechanisms which are responsible for the removal of particulate matter. This hypothesis was tested by the repeated injection of radiogold colloids in the same animal. Three consecutive doses at two-hour intervals were given to two dogs, whereas one dog received four such doses. There appeared no evidence of reticuloendothelial block in any of the animals. Thus the explanation of the curvature of the semilogarithmic plots in Fig. 1 must be sought elsewhere. A possible presence in our sols of small amounts of small particles, and the rapid initial removal of the large ones could account for the type of curve observed. The very flat portion of the curve as seen after the injection of large particles is in agreement with observations of Sheppard and associates.[2] They suggested that this may be due to the presence of some gold in the white cells which is not rapidly removed by the liver. We hope to explore this question with the autoradiographic technique.

The data obtained on tissues show that bone marrow takes up a relatively small amount of colloidal gold, and that both large and small particles are almost entirely removed from the blood stream by liver and spleen. Dobson and associates[1] found that in the rabbit small particles of zirconium and yttrium oxides are largely sequestered by the bone marrow. This apparent discrepancy could easily be the result of the use of different colloids and animals in the two studies.

SUMMARY

It has been shown that relatively large colloidal gold particles were removed from the blood stream of dogs at a more rapid rate than the smaller ones. The liver and spleen took up 80 to 90 per cent of the injected gold independent of particle size within the range studied.

The authors gratefully acknowledge the technical assistance of John C. Gallimore and are appreciative of the helpful discussions with Dr. C. W. Sheppard.

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THE PREPARATION AND PARTICLE SIZE MEASUREMENT OF MONODISPERSE RADIOACTIVE GOLD COLLOIDS

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INTEREST in the biological distribution of radioactive colloids is rapidly increasing for three reasons: (1) the use of commercially prepared colloids for therapy, (2) the increasing use by science and industry of the many radioactive elements having natural colloidal tendencies, and (3) the increasing realization that supposedly truly ionic solutions used for tracer work in many cases contain radiocolloids. Many properties of the colloidal particle and its development no doubt influence the pattern of distribution in the living organism. One property most readily studied is the particle size. Thus our first step in this general study was to investigate a method for particle size measurement simple enough to be carried out in a laboratory having a low-speed centrifuge. In this paper we shall present the results of an investigation of the centrifuge in the preparation of monodisperse radioactive gold colloids and the measurement of their particle size.

METHODS

Chemical Preparation of Gold Colloids.—Sixteen milligrams of gold foil containing 50 to 100 mc. of Au^{198} were dissolved in 1 ml. of concentrated HCl to which two or three drops of concentrated HNO_3 had been added. The solution was heated in a boiling water bath and taken down to dryness under vacuum. To remove the nitric acid, 1 ml. of concentrated HCl was added, and the solution was again dried under vacuum. This last step was repeated once more. Thirty-two milliliters of doubly distilled water, the second time in an all Pyrex still, were added to make a stock solution of $HAuCl_4$ containing 0.5 mg. of Au per milliliter.

To fifteen milliliters of the radiogold stock solution was then added 110 ml. of doubly distilled water, 3 ml. of 0.18N K_2CO_3, and 1 ml. of one-fifth saturated yellow phosphorus in ethyl ether. After standing fifteen minutes the solution was boiled for two to three minutes, and then oxygenated for one-half to one hour for the removal of the yellow phosphorus. These sols which were first prepared by Faraday[1] exhibited a wine-red color, were highly transparent, and showed no, or only a very faint, Tyndall effect. A discussion of the precautions necessary to obtain stable colloids can be found in standard texts of colloid chemistry, as well as in numerous published articles and reviews. In our experience the colloids made by the above method have shown excellent stability for as long as observed (three to four weeks).

In order to make gold particles of larger size, we added hydrogen peroxide and additional $HAuCl_4$ to the above gold sol, using the particles of the latter as nuclei for the formation

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of the larger particles. This procedure has been extensively investigated by Westgren[2] and others. The size of the resulting gold particles depends upon the relative number of gold nuclei in relation to the amount of gold chloride present for reduction. We found that a more stable gold sol was formed if the nuclei and H_2O_2 were mixed first and the $HAuCl_4$ was subsequently added dropwise. In contrast to the phosphorus reduced sols, the ones produced with H_2O_2 showed a poor stability, and they were ordinarily used within a few hours after preparation.

Measurement of Particle Size.—A number of different methods have been used for the measurement of submicroscopic particles. The electron microscope makes it possible to visualize small particles, but this instrument is not generally available. Brownian motion as observed under a dark-field, or particle count with the ultramicroscope has served to measure the size of various colloids. The limitation here, of course, is the lower limit of resolution which even for the ultramicroscope is only 75 Å. Unfortunately, the theory of light scattering by electrical conductors like gold has not been worked out to the extent that it could be used for the measurement of particle size. The osmotic pressure of gold colloids is too low to be accurately measured. Ultrafiltration has proved unsuccessful because of the strong absorption of gold particles onto the filter membranes. The only practical methods for the measurement of particle size which remain are the determination of the diffusion constant and sedimentation velocity. The ultracentrifuge has, of course, been extensively used for the measurement of molecular weights of large molecules. However, the particular advantage offered by this instrument, namely, the continuous recording of the sedimentation process, is made difficult by the slight difference between the refractive index of the solution and solvent in the case of gold colloids. Our attempt to measure the diffusion constant of colloidal gold with a Northrop diffusion cell[3] failed because of the strong adsorption of this material on the fritted glass disc.

A simple and practical method for the determination of particle size is that of Bechold and Schlesinger.[4] While they developed and used the method to measure the size of viruses, it seemed that the method could be readily adapted to measure the particle size of inorganic colloids such as gold. They used an ordinary low-speed centrifuge, and determined the sedimentation rate from the assumptions and the resulting equation given below:

The assumptions are: (a) that the colloid is monodisperse, (b) that the particles are spherical, (c) that enough vibrational agitation and convection currents are present to maintain a uniform concentration of particles throughout the supernatant, and (d) that the particles remain on the bottom of the tube once they have been deposited there.

In order to satisfy the fourth requirement they placed in the bottom of the centrifuge tube a layer of kieselguhr, which held the virus after it had once been thrown down.

Under these conditions the change in the concentration of the supernatant is given by

$$C = C_0 \, e^{-\frac{R}{h} Kt} \qquad (1)$$

$$K = \frac{2}{9} \frac{\rho^2(\sigma - \sigma')\omega^2}{\eta} \qquad (2)$$

C = concentration at time t
C_0 = concentration at zero time
R = distance from the axis of rotation to the top of the kieselguhr layer
h = height of the solution above the kieselguhr
ρ = radius of the particle
σ = density of the particle
σ' = density of the solution
ω = angular velocity (radians/sec) of centrifugation
η = viscosity (poises) of the solution

A number of materials were tested to serve as particle traps since the presence of trivalent ions in kieselguhr might possibly influence the aggregation of the gold. Kleenex was entirely unsuitable because it coagulated the colloid. No. 1 Whatman filter paper has the same tendency to a lesser extent. Absorbent cotton did not hold the particles tightly enough. No. 40 Whatman paper, when made into a pulp by boiling it with water, seemed to serve the purpose best. This paper did not visibly produce any coagulation of the colloid, but the fiber did seem to trap the colloid on its surface. The necessity of using a layer of filter paper on the bottom of the tube was demonstrated by centrifuging the same colloid with and without a particle trap. The concentration of the colloid centrifuged with filter paper decreased 50 per cent in radioactivity in one hour, whereas the same colloid centrifuged in a plain tube lost only 1 per cent.

The assumption that no concentration gradient exists in the supernatant fluid was tested after a five-hour centrifugation period by external counting of the centrifuge tube filled with radioactive colloid. By counting at different heights above the filter paper it was found that the concentration of colloid was the same at all levels of the tube.

Another test of the method was made in the following way. The same colloid was centrifuged in a 10 ml. Lusteroid tube ($R = 18.5$ cm., $h = 8$ cm.) at 3,500 r.p.m. and in a 100 ml. Lusteroid tube ($R = 27$ cm., $h = 13.5$ cm.) at 2,000 r.p.m. The curve obtained from the first set of conditions gave a particle radius of 4.45 mμ. The second set of conditions gave a particle radius of 4.47 mμ. This shows that within the above limits the results do not depend on the length and cross section of the tube or the speed of rotation.

In Fig. 1 some typical centrifugation curves are presented. The numbers at the end of each curve indicate the measured diameter in mμ. The monodisperse colloids give straight lines on a semilogarithmic plot. To make certain of the monodispersity, one should continue to centrifuge until practically all particles have precipitated. The curves labeled "polydisperse" show that an appreciable percentage of larger particles were present. The degree of polydispersity can be estimated from the extrapolation of the late portion of the curve back to zero time. The ratio of this extrapolated value and the zero time concentration is the fraction of the colloid which is present in the form of small particles. Approximately 75 per cent of those colloids presented by the two polydisperse curves of Fig. 1, consists of 9 mμ and 10.5 mμ particles. Thus a monodisperse colloid can be prepared by a one- to two-hour centrifugation. Consequently the same method can be used for the *preparation* as well as for the *measurement* of particle size of monodisperse gold colloids.

The top three curves of Fig. 1 give some idea about the sensitivity of the procedure. Three sols with a particle diameter difference of only 15 per cent are represented by three curves with distinctly different slopes. The next two curves below show the effect of speed of centrifugation. A particle "A" with twice the diameter of "B" will give the same slope as "A" at half the speed of rotation. The lower two curves show the effect of gelatin upon the sedimentation rate. The same colloid was centrifuged with and without added gelatin. The gelatin-stabilized colloid settles much slower than the unstabilized colloid. The probable reason for the difference is an increased viscosity of the liquid and a decreased density of the gold-protein complex.

A further check on the reliability of the particle diameter obtained by the method was made by comparing the diameters of a number of gold particles where the larger size was grown from the smaller by the addition of $HAuCl_4$ and H_2O_2. To a mixture of radioactive gold nuclei of known particle size and a few drops of H_2O_2 was slowly added a known amount of $HAuCl_4$ under constant shaking. The resulting sol was centrifuged and the particle size measured.

Where Au_n = amount by weight of gold in nuclear fluid
Au_a = added gold by weight (as chloride)

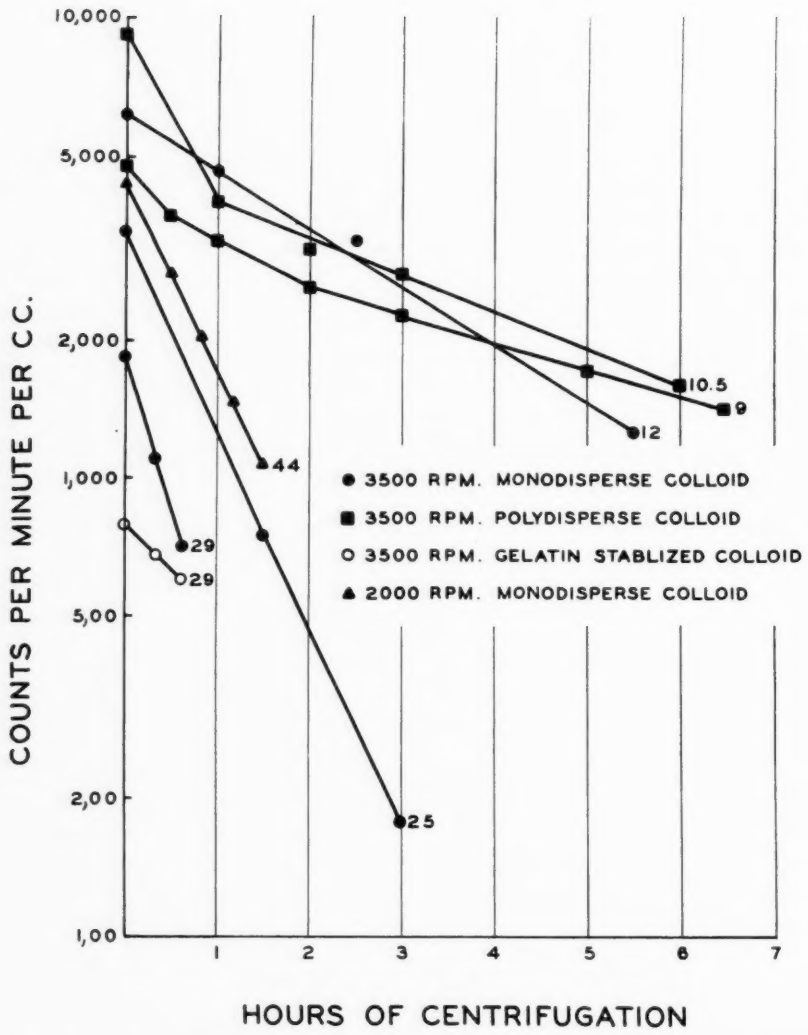

Fig. 1.—A semilogarithmic plot of the concentration of Au^{198} during centrifugation. The numbers on the right of the curves are the measured particle diameters.

d = density of gold
r_1 = radius of gold nuclei
r_2 = radius of final gold particles

$$\text{No. of gold nuclei} = \frac{Au_n}{4/3\ \pi\ r_1^3\ d} \quad (3)$$

$$\text{No. of final gold particles} = \frac{Au_n + Au_a}{4/3\ \pi\ r_2^3\ d} \quad (4)$$

If each gold nucleus produces one larger gold particle and no additional nuclei are formed during the growing procedure, then the number of particles must remain constant. This yields the following relation:

$$r_1 = r_2 \sqrt[3]{\frac{Au_n}{Au_n + Au_a}} . \quad (5)$$

In Table I we have used Equation 5 to calculate the diameter of the smaller particles from the next larger ones.

TABLE I

	SMALL GOLD PARTICLES	0.5 MG. AU^{+++} $\xrightarrow{H_2O_2}$	INTERMEDIATE GOLD PARTICLES	1.5 MG. AU^{+++} $\xrightarrow{H_2O_2}$	LARGE GOLD PARTICLES
Measured diameter mμ	8.9		29		44
Calculated diameter mμ	12.8		28		44
No particles per milliliter	6.7×10^{12}		2.1×10^{12}		2.3×10^{12}

This table shows that if we assume the measured diameter of the 44 mμ gold particles to be the "true" diameter, then the measured diameter of 29 mμ agrees quite closely with the calculated one (28 mμ). However, in the calculation of the small particles' size a discrepancy occurs in the measured and calculated diameters. The measured diameter is less than the calculated diameter and consequently it appears that there are more small particles than large ones. In an additional experiment nuclei of 10.6 mμ diameter were grown with 1.9 mg. of Au to particles of 47 mμ diameter. Again the number of nuclei was found to be three times as large as that of the larger particles.

There are several possible explanations for the discrepancy. It is possible that the filter paper is not an efficient trap for the smallest particles. The discrepancy could be entirely explained if 50 per cent of the particles that sediment during a given time were released back into the supernatant. Another explanation could be that nuclei of less than ten mμ diameter coalesced during the growth of larger particles. A third mechanism, that of hydration to decrease the density of the particles, could explain the discrepancy only in part. A rough calculation shows that in order to lower the density sufficiently a water envelope of more than ten layers of water molecules would be required, which is very unlikely.

If the diameter of the smallest particles is recalculated on the assumption that the true number of particles per milliliter is 2×10^{12}, instead of 6.7×10^{12}, one finds that the small particles whose measured diameter is between 9 and 11 mμ may in reality have a diameter of 13 to 16 mμ. It should be noted that this is not serious for the interpretation of results to be presented in the following paper.

SUMMARY

A method is presented whereby monodisperse radioactive gold sols can be prepared and their particle size measured by means of a low-speed centrifuge. The procedure is simple enough to be used routinely in the characterization of radioactive materials before their administration to patients or animals.

The authors gratefully acknowledge the technical assistance of John C. Gallimore.

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MENTAL SYMPTOMS FROM MYXEDEMA

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IT IS a well-known and accepted fact that personality changes are common in persons with myxedema. These changes are often obvious, and many physicians have seen patients whose social conduct, work habits, and family relations have shown pronounced deviation from their normal behavior pattern. The response of such a person to the proper use of thyroid therapy is sometimes dramatic. While most physicians know that dullness and poor memory may accompany myxedema, few realize that frank psychosis may occur. It is the purpose of this paper to report two patients with myxedema whose personality changes were so severe that they were committed to mental institutions with diagnoses of definite psychoses.

The medical literature has little to say concerning myxedema accompanied by severe mental symptoms. Most texts usually omit any mention of personality changes except to state that such patients may be sluggish or somewhat slow in their mental processes. Asher[1] points out the sparsity of information on this subject and reports on fourteen patients on mental wards in English hospitals who were cured of their "insanity" by the use of desiccated thyroid. All of his cases were shown to be myxedematous. Ruhberg, Stoll, Zondek and Wolfsohn,[3, 4, 7] and others have also reported isolated cases of similar circumstance.

The committee of the Clinical Society of London reported in 1888, after five years of study, that "insanity" was not uncommon in myxedema. They further reported that the manifestations followed no set pattern, but that reactions tended to follow previous personality patterns of the individual patients. It was found, however, that melancholia was frequent, as was mania, and that patients showed traits of marked suspicion and self accusation.

Differential diagnosis is somewhat difficult at times. Asher points out, as does Youmans and Riven,[6] that *mental and nervous symptoms may dominate the picture before myxedematous features appear.* In cases of severe mental disturbance the value of physical examination and determinations of basal metabolic rate are of questionable value because of poor cooperation, misleading responses, and undue excitement.

CASE REPORTS

CASE 1.—M. H., a 26-year-old white married woman, was admitted to a Michigan state hospital with a diagnosis from the certifying physicians of manic depressive state. She allegedly had been well until five months prior to admission when she consulted her family physician because of her discovery of a small nodule in the region of the thyroid. She was sent by her physician to a regional hospital center where a biopsy of a cervical node was performed and a pathologic diagnosis of metastatic carcinoma was made. Following other

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studies, including a basal metabolic rate of plus 1 per cent, a radical neck dissection with total thyroidectomy was performed. The preoperative impression of primary carcinoma of the thyroid was confirmed. Her postoperative course was uneventful except for minimal signs of tetany which were easily controlled with calcium administration. She was sent home on dihydrotachysteroi and calcium and did quite well for several weeks. She was taught to run her own Sulkowitch urine tests, and serum calcium determinations were done occasionally and were always at satisfactory levels.

Shortly following operation she was told that she had had a malignant lesion, but was reassured by the attending surgeons that her chances for complete and lasting recovery were excellent. However, she soon began to show signs of concern and, according to the family, became quite moody and brooded considerably over this diagnosis. At no time during the five-month interval between surgery and her admission to this mental institution did she have a basal metabolic rate, serum cholesterol, or any other metabolic study except the serum calcium determinations. During this time she became progressively more morose, inactive, uncommunicative, and, as her family described it, "extremely depressed." At the suggestion of the family physician, and with the consent of the patient's family, committment proceedings were undertaken and she was admitted to this mental institution five months following surgery.

She was seen by a resident psychiatrist who tentatively agreed with the admitting diagnosis of manic depressive state in depressive phase. She was seen two days later by the medical consultant at the hospital in regard to her parathyroid medication. It was his impression at the time that the thyroid status should be investigated, despite the fact that she was in no way a typical case of myxedema. She demonstrated lack of interest in her surroundings; guilt feelings about her poor housekeeping and about absence of sexual interest in her husband. In connection with the latter she had strong suspicions regarding her husband's fidelity.

Physical examination showed a pale, dry skin and possible decrease in thickness of the eyebrows. The blood pressure was 95/65, right arm reclining, and the pulse was 60 per minute. Findings otherwise were considered to be normal.

The basal metabolic rate at the time of admission was minus 39 per cent, and the serum cholesterol was 395 mg. per cent. An electrocardiogram was not remarkable except for generally decreased amplitude. Routine blood and urine studies were normal. A serum calcium could not be done at the institution laboratory.

The patient was started on desiccated thyroid in the amount of 0.1 Gm. daily and was continued on parathyroid replacement which consisted at that time of dihydrotachysterol in the amount of 2.5 mg. twice weekly and supplemental calcium salts. During the first two weeks in the hospital the patient received no psychiatric treatment, but continued to take the medications listed above. During her early days in the institution she presented typical symptoms of depression. Her time was spent sitting in a chair, taking an interest in nothing, and crying frequently. She refused occupational therapy, and declined to help with housekeeping duties on her ward. This attitude began to change, however, after only ten days of thyroid administration. She became more alert and gradually developed an interest in her surroundings and the proffered activities. Interview by the ward psychiatrist at the end of two weeks also showed impressive improvement in her general condition. A repeat basal metabolism at that time was minus 17 per cent. A repeat cholesterol was 290 mg. per cent. She was continued on desiccated thyroid for another two-week period during which time no psychotherapy was given. At the end of that time, one month after the beginning of treatment with desiccated thyroid, the basal metabolic

rate was minus 8 per cent, and the cholesterol was 225 mg. per cent. Her pulse rate was then 70 per minute, and the blood pressure was 105/70, right arm reclining. Despite a definite increase in appetite, she had lost 4 pounds in weight.

The patient was then presented in psychiatric staff conference and all present agreed that she was mentally normal in so far as could be determined. At this time she was taking a full and active part in occupational therapy courses, helped on the ward, and worked in the cafeteria during meal hours. Above all, she had an excellent insight into her past depression which is not a part of the picture of psychotic depressions. Neither did she show any symptoms of being in a manic phase, which might be expected to follow depression in a true manic depressive state. It was the impression of all concerned with her case that the past symptoms likely were due to a metabolic disturbance, i.e., myxedema. She was discharged to her home several weeks later, apparently normal in all respects. She is being followed in the outpatient department by the hospital psychiatrist and now, six months after discharge, is still doing very well. Reports from her family state that she seems completely adjusted. Her latest basal metabolic determination, nine months after start of treatment, was plus 10 per cent, and she continues on 0.1 Gm. of desiccated thyroid daily.

CASE 2.—H. D., a 35-year-old white married man, was considered a very promising business executive until six months prior to his admission to a mental hospital. However, in the several months preceding his admission, his behavior, appearance, and work efficiency so deteriorated that he was suspected of drinking to excess, using drugs, or being mentally ill. Various treatment by his physician failed to improve this condition. Eventually, he was diagnosed as being in an acute depressive state and was committed to a mental institution on a temporary court order.

Again myxedema was considered as a diagnosis and confirmed by a basal metabolic determination of minus 41 per cent and a serum cholesterol of 405 mg. per cent. Desiccated thyroid was prescribed in the amount of 0.1 Gm. daily, and the patient showed rapid response, with disappearance of all symptoms at the end of eight weeks. Basal metabolic rate and serum cholesterol level both returned to within normal limits after twenty weeks of therapy. The patient is now back at his former position and doing excellent work while continuing on the above dosage of desiccated thyroid. This patient also failed to show either typical myxedematous appearance or other physical signs. His general physical condition would have been considered to be within normal limits.

DISCUSSION

As mentioned earlier, it is well known that personality changes occur in myxedema. Nor is it unusual for persons with hypothyroidism to be seen by private psychiatrists because of their behavior changes which are suspected as being on a psychotic basis. Such physicians not uncommonly observe persons with depression, paranoid ideas, and guilt feelings who respond dramatically to thyroid therapy. One such person, seen in private practice by E. Wright, was a young married woman with a very compulsive, driving personality. With the onset of thyroid deficiency she found that she lacked the energy, enthusiasm, and ambition to continue her very active life, which fact caused her to

feel quite depressed and also brought forth guilt feeling because she was unable to maintain her home as she would have liked. She further accused her husband of infidelity, which was not the case, possibly because she herself had no sexual interest in him. Thyroid therapy promptly relieved all symptoms.

It is admitted that the patients reported here were not undeniably proved to have had their symptoms on a basis of thyroid deficiency alone. To complete the postulates necessary for final proof, one would have had to remove such patients from thyroid to see if a remission occurred and then to treat them again. This was considered, but the potential danger to the patient inherent in such a program did not seem to justify this test. In view, however, of the prompt improvement on desiccated thyroid without psychotherapy, and in the absence of other causative factors which might have caused true psychotic episodes, all concerned were agreed that thyroid deficiency was the most likely cause of the condition. Asher[1] reached the same conclusions with his patients who responded only with thyroid after many other treatments had failed.

SUMMARY

Two cases of "mentally ill" patients are presented. They were promptly relieved of their psychoses by thyroid administration, and are considered to have been myxedematous. Physicians should keep in mind the possibility of frank or masked myxedema in patients with drastic personality changes or in those suspected of having a true psychosis.

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EFFECTS OF DIALYZER CONSTRUCTION ON TRANSFER RATES

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INTRODUCTION

TO EVALUATE the efficiencies of several structurally different dialyzers, transfer rates of the sugar, dextrose, were observed under varying conditions. Dialyzers have proved useful in chemistry for separating substances of different molecular weights and in medicine as artificial kidneys for removing waste materials from blood.

The phenomenon of dialysis was discovered by T. Graham[1] in 1861 using sheets of parchment, and at that early date he successfully dialyzed urine to separate its crystalloid components. Early investigators[2] met with difficulties due to nonuniform membranes, and it was not until the development of cellophane sheets, and later tubing, that the application of this phenomena became common. With the development of suitable blood anticoagulants, medical dialyzers soon appeared.

Transfer rates of single-pass continuous dialyzers have been reported[3] with information applicable to such processes as beet-sugar refining and recovery of caustic soda from hemicellulose for the chemical industries.

Transfer rates of substances of interest in medical dialyzers have been reported by Alwall and associates,[4] Merrill and associates,[5] and Kolff,[6] but it was felt desirable to determine batchwise transfer rates in vitro for dialyzers of different designs and to develop a standard test for comparison of instruments and evaluation of design variables.

Recently Wolf, Remp, Kiley, and Currie[7] have published a concise performance study on the Kolff-type rotating dialyzer which tabulates transfer rates of many substances in that particular type, and observes several operating parameters peculiar to that instrument which are variant with dialyzer design.

The two medical dialyzers studied here were both built to the design of Dr. Willem J. Kolff of the Cleveland Clinic, who was one of the first investigators to develop the first clinical instruments. One unit employed the countercurrent flow principle with the dialysate solution (solution initially containing the substance to be recovered) flowing through a flattened cellophane tube surrounded

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in a small chamber by the rinsing solution (solution into which the substance will diffuse) flowing in the opposite direction. A second unit employed a rotating drum on which the cellophane tubing containing the dialysate solution was wound in a spiral. This drum was partially immersed in a tank containing the rinsing solution.

A simplified vertical dialyzer consisting of a length of cellophane tubing containing the dialysate solution suspended in a glass tube containing the rinsing solution was constructed to evaluate the technique and to check osmotic pressures.

Dextrose was chosen as the diffusing substance because of a molecular weight similar to urea and creatinine and ease of measurement of concentration due to its optical activity.

EXPERIMENTAL

Membrane.—The dialyzing membrane was in all cases 20/32 inch inner diameter inflated, Visking tubing manufactured by the Visking Corporation of Chicago, Ill. This tubing is produced by the regeneration of cellulose from viscose. Pore diameters of cellophane sheets produced by similar means have been reported as between 25 and 50 Angstrom units.[3] This would preclude diffusion of most substances of molecular weights greater than 100,000, and depending on the structure of the molecule, the weight limit may be much lower. In a study of dialysis of substances known to form colloidal sols through Visking tubing, Thor[8] has reported that Bacto peptone and Dextrine diffused, but that egg albumin sol gave only a doubtful trace indication of diffusion at pH 8.7 and serum albumin sol no indication. The diameter of a dispersed particle of egg albumin is 40 Å,[9] and this may support the above conjecture as to pore size. Recently, Philipp and Björk[10] have reported a pore radius of 24.5 Å for No. 600 gel cellophane.

Osmolarity.—Osmotic equilibrium was approached by adding 5 Gm. of magnesium sulfate per hundred milliliters of distilled water (rinsing solution) to balance the osmotic pressure of 20 Gm. of dextrose per hundred milliliters of dialysate solution. Due to the difference in diffusion coefficients between the two, the rinsing solution varied slightly from the isotonic condition during the runs, but volume differentials were not excessive. The reproductibility of the experiment was first checked by repeating identical runs on a simple vertical dialyzer shown in Fig. 1.

Preparation of Solutions and Polarimeter Technique.—The dextrose solution was made by adding 300 Gm. of dextrose to approximately 1,200 c.c. of distilled water. When the dextrose was fully dissolved the volume was measured and distilled water added until the total volume reached 1,500 c.c. Two or three drops of $HgCl_2$ were added to the dextrose solution to inhibit bacterial action. Dextrose solutions were always made up the day before they were to be used, as it was found that it took some time for the optical activity of the solution to reach equilibrium. The $MgSO_4$ was made by adding 20 Gm. of $MgSO_4$ for each

100 c.c. of the final volume to a volume of water somewhat less than the desired final volume. After the $MgSO_4$ crystals were dissolved, water was added until the desired volume was reached.

At regular intervals the quantity of dextrose which had dialyzed through the membrane was determined by means of polarimetry. The sample of the dextrose solution was taken from the system after it had passed through the dialyzer and before it was returned to the reservoir. The polarimeter sample holder was filled with the dextrose solution, and the rotational value was read from the polarimeter. By comparing this value with the rotational value of two

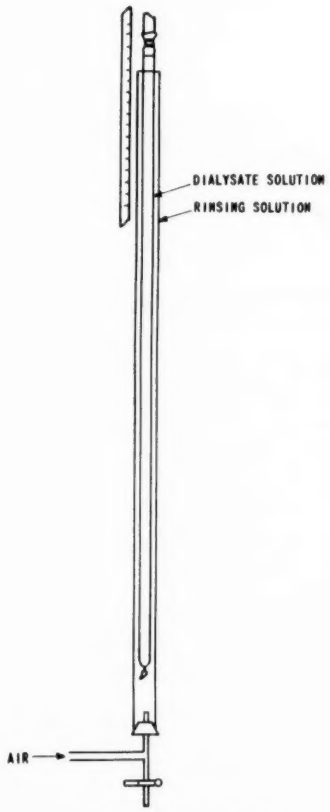

Fig. 1.—Vertical dialyzer.

solutions of known concentration, the weight of the dextrose in the solution was computed. In this case the reference solutions were distilled water and a solution of dextrose having a concentration of 20 Gm. of dextrose per 100 c.c. of solution. Readings for each sample were taken three times, and the average reading was used for computations. Data from a sample run are tabulated in Fig. 8. Computed completion was calculated from the condition of diffusion equilibrium of the sugar. After an initial pH shift from 5.5 to 6.2, the dextrose solution remained in the range of pH 6.2 to 6.44 during the tests.

Vertical Dialyzer.—A tube of Visking Cellophane membrane, 5/8 inch outer diameter inflated, was sealed at its lower end to contain the dialysate solution. This in turn was suspended in a glass tube 1 inch inner diameter, thus containing the rinsing solution in an annular chamber. Agitation was secured by bubbling air slowly through the rinsing solution. The bubbles massaged the Visking during their passage up through the fluid. Samples of rinsing solutions were drained off periodically to check optical activity, and then returned to the chamber.

Results of these tests showed the experimental variation to be within ± 11 per cent. Reproductibility is within limits imposed by variations in membrane samples, rates of agitation, ambient temperature variations, and other experimental errors.

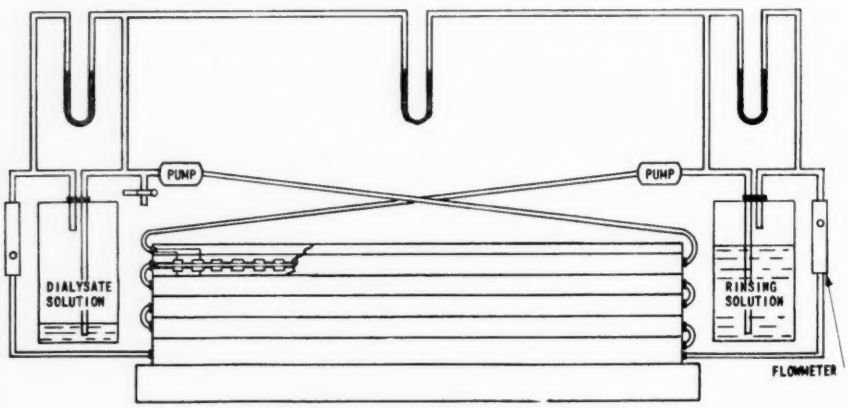

Fig. 2.—Flat-plate dialyzer.

Flat Plate Dialyzer.—Constructional details of a flat plate dialyzer developed by Kolff are shown in Fig. 2. In this type, the dialyzing membrane, again Visking tubing 5/8 inch outer diameter inflated, was used, although the unit had been designed for a slightly larger tubing (34/32 inch flat Visking).

The membrane was suspended in a horizontal position in Lucite blocks with raised teeth to permit turbulent flow of the rinsing solution on top and bottom of the flattened tubing. The dialysate solution was passed through the tubing countercurrent to the surrounding rinsing solution. Manometers were attached to the system to measure pressure drops of dialysate and rinsing solutions through the dialyzer and also intraluminal pressure differentials. Rinsing solution flow rates were 400 milliliters per minute in all cases. The cross-sectional area of the channels was approximately 5.26 square centimeters. Five channels stacked one above the other were connected in series.

Rotating Dialyzer.—Fig. 3 shows construction of the Allis-Chalmers Kolff-type rotating medical dialyzer employing 165 feet of 5/8-inch Visking tubing as the dialyzing membrane wound in a spiral on a stainless steel drum which is

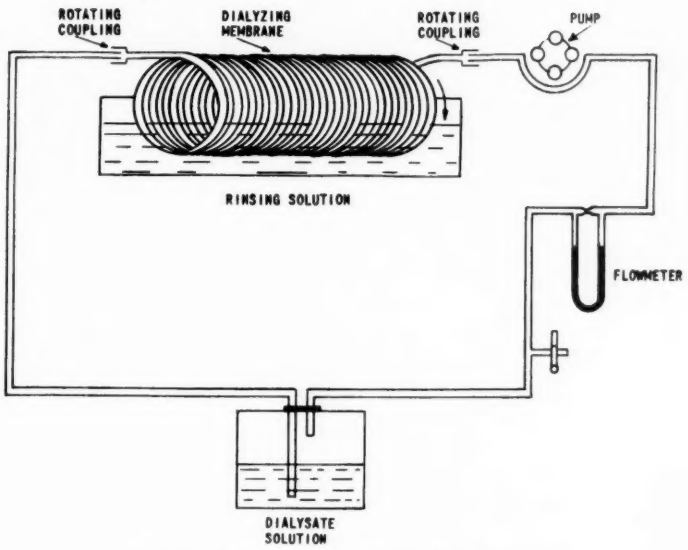

Fig. 3.—A-C artificial kidney, Kolff type.

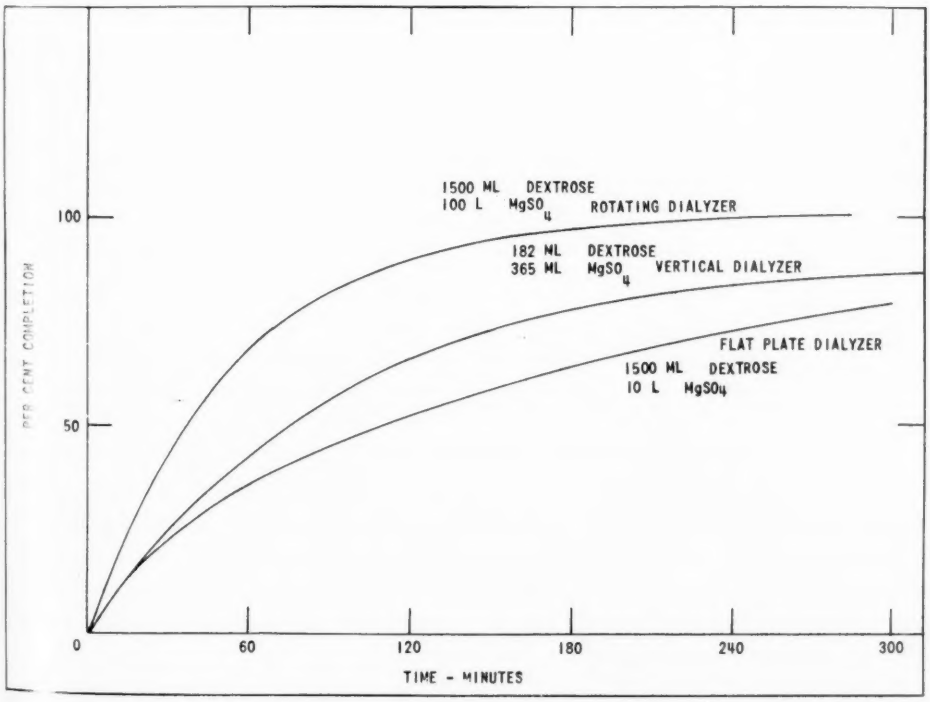

Fig. 4.—Comparison of per cent completion.

rotated in the rinsing solution. The dialysate solution travels through the membrane tubing by the action of the Archimedes spiral, and is then pumped back into a reservoir. The reel diameter is 42 centimeters, and 34.2 per cent of its circumference is submerged.

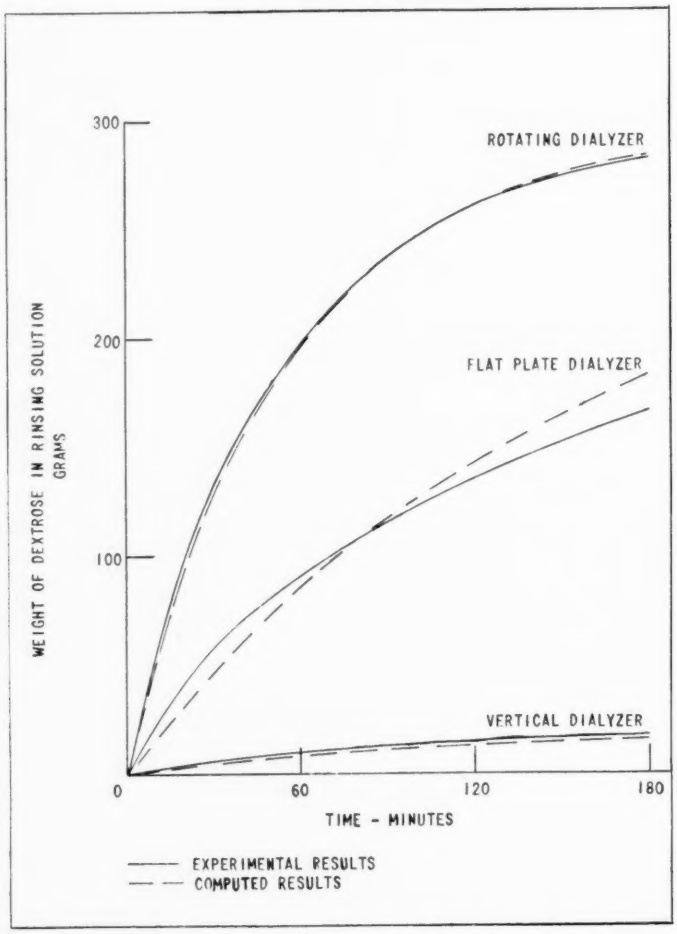

Fig. 5.—Calculated and experimental rates for three dialyzer types.

Relative Efficiencies.—The relative efficiencies of the three types of dialyzers are shown in Figs. 4 and 5. It will be noted that dialysate solution volumes for the rotating and flat-plate dialyzers were identical. In all cases, dextrose initial concentrations in the dialysate solution and magnesium sulfate in the rinsing solution were the same.

The data, as expected, varied as a natural logarithmic function similar to variations encountered in growth and decay functions such as radioactive decay.

This was because the diffusing substance created successively smaller concentration differentials until equilibrium was reached. This permitted use of an exponential equation of the type:

$$N = N_0 e^{-\mu t}$$

From the data average rate coefficients μ were calculated for the different types of dialyzers for use in the formula:

$$C = C_e (1-e^{-\mu t})$$

Where

C = Concentration of diffusing substance in rinsing solution at time t.
C_e = Equilibrium concentration
μ = rate coefficient
t = time

TABLE I

	μ (MIN.$^{-1}$)	T (MIN.)
Rotating dialyzer	0.0169	41
Flat plate dialyzer	0.0068	102
Vertical dialyzer	0.0094	74

The data give empirical rate coefficients as shown in Table I.

The half time T is the time in which half of a given mass of dextrose will be transferred from dialysate solution to rinsing solution. The half time T relationship between the rotating dialyzer and the flat plate dialyzer for dextrose is an indication of relative rates of transfer of other substances.

A comparison of the rate coefficients and half times of the other two types with the vertical dialyzer must take into consideration the fact that the dialysate solution volume is only 200 ml., as compared with 1,500 ml. for the others. This means that much less mass of material can be transferred through the wall per unit time, as evidenced by Fig. 5.

Calculated Transfer Rates.—Fig. 5 shows rates of mass transfer in the dialyzers for three-hour runs with experimental data plotted in conjunction with curves calculated from the empirical rate coefficients listed in Table I. Close agreement is shown. Dialysis lasting longer than three hours showed a departure from the exponential with curves flattening out at lower levels than those calculated. Since three-hour runs accounted for transfer of over 50 per cent of the mass of the dialyzing substance for the flat plate dialyzer and 90 per cent for the rotating model, averaging at the rate coefficients over this period was felt to be adequate.

Effects of Membrane Area.—The effects of area of the dialyzing membrane on rates of dialysis in the flat plate dialyzer are shown in Table II. Calculation of the rate coefficients of the unit with varying membrane area shows close to a linear relationship. Varying numbers of stages were employed with the unused channels blocked off.

TABLE II

AREA (CM.^{2})	NUMBER OF STAGES	μ MIN.$^{-1}$	X $\times$ 10^{-6} CM.$^{-2}$ MIN.$^{-1}$
2045	5	0.00673	3.29
1227	3	0.00429	3.49
409	1	0.00140	3.43
			X average = 3.40

The rate coefficient of this dialyzer can be expressed then as $\mu = XA$ (where A = area) with X average being used for the calculation.

To achieve a transfer rate coefficient comparable to the rotating type at identical flow rates (in this case 200 milliliters per minute) if the extrapolation to greater areas is valid, which it would appear to be, an area of 4,930 square centimeters would be required. This compares favorably with the 15,863 square centimeters used in the rotating type, pointing to the efficiency of the countercurrent system.

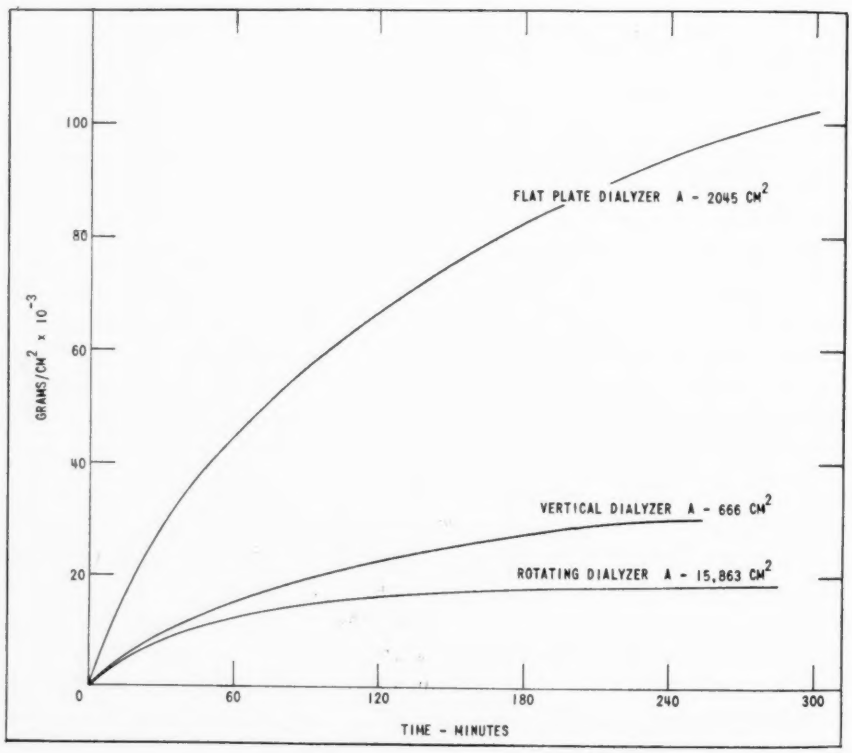

Fig. 6.—Mass transfer per unit area vs. time.

The relative efficiencies of the three dialyzers per unit area of membran are shown in Fig. 6. This emphasizes the fact that the countercurrent flo type is by far the most efficient. The small vertical type with only gentle agita tion of the solutions is more efficient than the rotating drum type with muc

more agitation. This suggests that the area on the drum not immersed in the rinsing solution is not effective in dialysis. Previously it was felt that with a rotation of eighteen revolutions per minute of the drum (a speed which produces smooth action of the Archimedes spiral in fluid transfer, although slightly greater speeds of rotation have been used) the upper portion would be sufficiently wet with rinsing solution for that membrane area to act as if immersed in the solution.

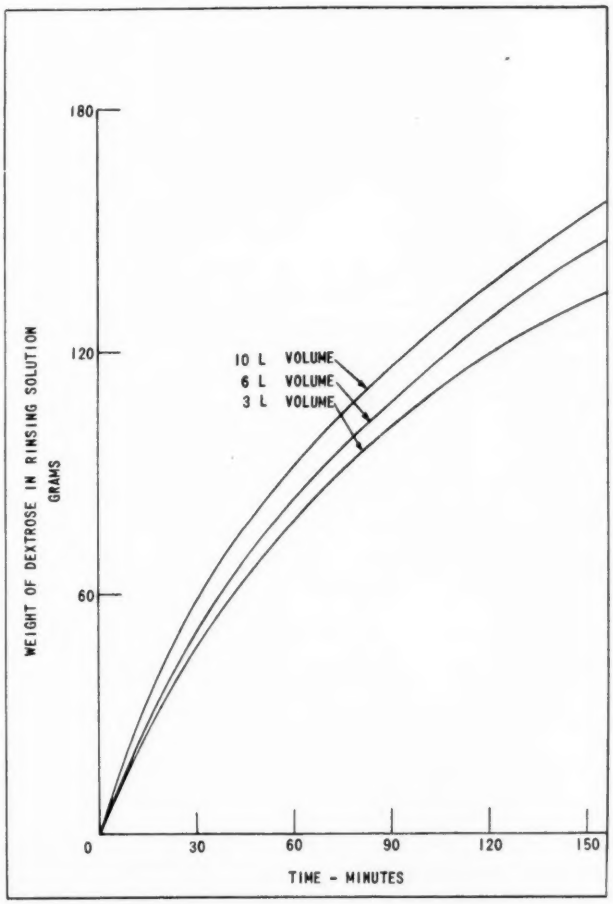

Fig. 7.—Effect of varying rinsing solution volumes on rates of dialysis.

The significance of the data shown in Fig. 6 should be emphasized. It is of primary importance in designing dialyzers to choose a method producing highest transfer rates with minimum size and maximum ease of operation. Over-all size is generally proportional to dialyzing area, and Fig. 6 shows that for one-hour operations the flat plate dialyzer is 3.75 times more efficient than the rotating type per unit area of membrane, 4.18 times after two hours, and 4.55 times after three hours.

This points to the efficiency of countercurrent flow and the necessity of further investigation of increased agitation at the membrane faces to secure even more rapid transfer rates. Total immersion is also indicated by comparison of the rotating with both other types.

Intraluminal Pressure and Dialysate Flow Rates.—Two additional phenomena of extreme importance to dialyzer design were studied, namely, effects of intraluminal pressure and dialysate solution flow rates.

DEXTROSE

TIME	° ROTATION				% COMPLETION	WT. OF DEXTROSE IN RINSING SOLUTION
Min.	R.	R_2	R_3	Ave. R.	Computed	
0	21.5	21.8	21.4	21.6	0.0	0
30	16.6	16.5	16.6	16.57	26.7	68
60	14.75	14.9	14.4	14.68	36.2	96
75	13.9	13.75	13.85	13.83	41.2	108
120	11.2	11.3	11.25	11.25	54.8	144
150	9.5	9.6	9.7	9.6	63.7	166
180	8.6	8.45	8.75	8.6	68.8	180
210	8.2	8.25	8.1	8.18	71.2	187
240	7.25	7.6	7.3	7.38	75.3	198
270	7.0	6.9	6.7	6.9	77.9	204
300	6.0	6.1	6.25	6.12	82	215

COMPUTED COMPLETION - 2.61 gm/100 cc.= 2.72 °Rot.

Fig. 8.—Typical data sheet.

Several investigators have felt that rates of transfer were accelerated by applying a pressure differential from the dialysate solution to the rinsing solution. In the experiments with dextrose, differentials from 0 to 200 mm. Hg were applied in the flat plate dialyzer, with no apparent increase in transfer rates. There seems no reason to believe that the transfer rates of other materials in solution would be affected by pressure differentials, the rates being dependent basically on concentration gradients and diffusion coefficients. Fluid transfer through the membrane was not obviously influenced by pressure differentials.

Measurements of dextrose transfer rates at varying dialysate solution flow rates showed that in the case of the flat plate dialyzer saturation of the transfer rates had been reached below the lowest flows measured, 25 milliliters per

minute, and nothing was gained by increased flow rates; indeed, slight decreases in transfer rates were observed. It must be emphasized that this was a batchwise measurement, not a measurement of the ratio of dialyzer outlet concentration to inlet concentration. This ratio, similar to the extraction ratio, varies directly with flow rates, relatively more substance being removed with slower flow rates.

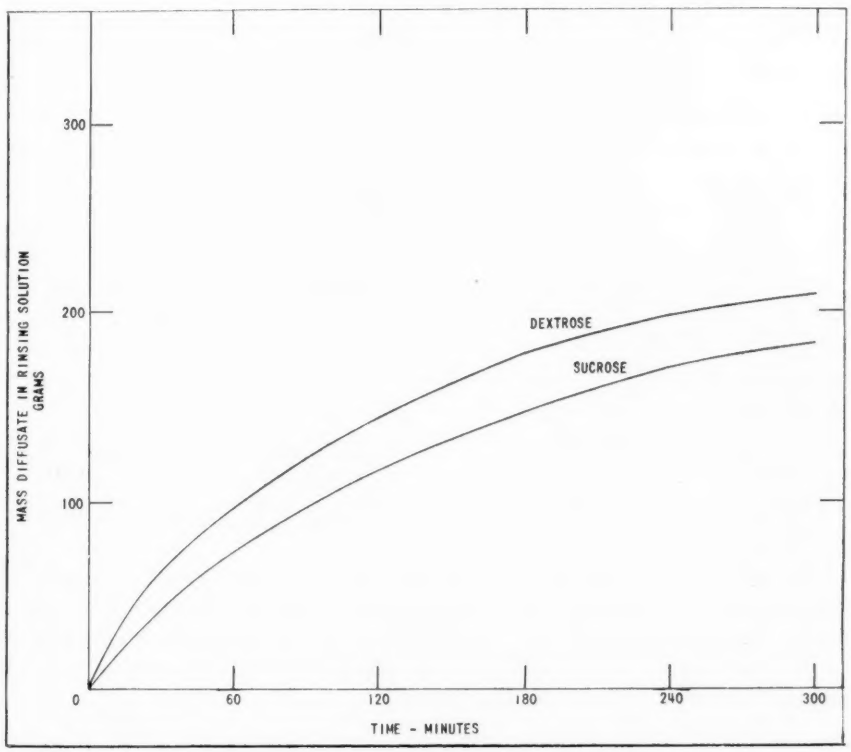

Fig. 9.—Comparison of rates of dialysis for dextrose and sucrose.

One of the important factors in batchwise dialysis is the number of passes the total dialysate volume makes through the instrument. If all of the dialysate solution has circulated several times during the period, increased flow rates would have been of little advantage. This means that for any given dialysate solution volume, there is a flow rate for that solution in batchwise dialysis above which no increase in transfer rates are obtained. This may be considered the saturation flow rate.

Presumably this same saturation flow rate phenomena is operative in other dialyzer designs.

Volume Ratios.—Variations in transfer rates with changes in rinsing solution are shown in Fig. 7. A point of diminishing return is quickly reached in

increasing the ratio of rinsing solution to dialysate solution. The rate coefficient formula:

$$c = C_e\ (1-e^{-\mu t})$$

The above was extended to include volume relationships as follows:

$$M = CV\ \left(\frac{V^1}{V^1 + V}\right)\ (1-e^{-\mu t})$$

M = Mass of dialyzed substance transferred to rinsing solution at time t (grams), where c = initial concentration of substances in dialysate solution (grams liters^{-1})

V = Dialysate solution volume (liters)
V^1 = Rinsing solution volume (liters)
μ = Rate coefficient (minutes^{-1})
t = Time (minutes)

This formula was used to calculate transfer gains achieved by increasing rinsing solution volumes (dialysate solution volume was constant at 1.5 liters). It is evident that for dialysis of three hours or less, little is gained by a rinsing solution volume in excess of four times the dialysate volume. This is dependent on concentration gradients or rate of rinsing solution saturation. This is a crude approximation as μ varies with volume ratio.

The formula was checked experimentally but does not agree too closely for the flat plate type; however, it does serve to indicate that indefinite increase of rinsing solution to dialysate solution volume ratio is not in order in short period batch dialysis.

Rates of Various Substances.—No investigation was made of the transfer rates of the many substances other than dextrose separable by dialysis, with one exception. A comparison of the typical rates of two substances, i.e., dextrose and sucrose, is shown in Fig. 9. This relationship is probably primarily dependent on the molecular diameter of the hydrated molecule.

The larger molecule sucrose, a disaccharide, diffused into the rinsing solution more slowly than the monosaccharide dextrose.

Rates of transfer of materials of medical interest through the rotating dialyzer have been reported.[7]

SUMMARY AND CONCLUSIONS

The two medical dialyzers studied represent a marked contribution to medical physics of their designer, Dr. Willem J. Kolff of the Cleveland Clinic.

This investigation studied these two units in an attempt to isolate the variables affecting efficiencies of dialyzers in general, and point out those factors which will be important in design of future units.

The conclusions are as follows: (a) for a given area of dialyzing membrane, the countercurrent flow type appears most efficient (Fig. 6). (b) Of the three dialyzers studied in their normal operating condition, the rotating type transfers the most diffusate in a given time (Fig. 5). (c) High dialysate

solution flow rates may not be required. This must be modified according to dialysate solution volume and time of dialysis. (d) No increases in transfer rates are apparent with intraluminal pressure differentials of up to 200 mm. Hg. (e) Calculation of rinsing solution volumes should consider dialysate volume, dialyzer transfer rates, and time of dialysis. Reasonably rapid transfer rates can be obtained without excessive rinsing solution volumes (Fig. 7). (f) In the flat plate dialyzer, transfer rates vary linearly with area. (g) Empirical rate coefficients allow calculation of mass transfer during dialysis (Fig. 5).

Further investigation may relate transfer rates to molecular weight and size to allow prediction of these rates without experimental measurements.

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THE HISTOCHEMICAL CHARACTERIZATION OF ARTICULAR CARTILAGES OF POLIOMYELITIS PATIENTS

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IN A previous paper,[1] the histochemical characterization of normal hyaline cartilage obtained from experimental animals was described. It is proposed in a series of additional studies to characterize hyaline cartilage of human patients. These cartilages were obtained from a number of patients with varied diseases. Regrettably, we do not yet have data on hyaline cartilage from normal human subjects which would provide a base line from which to measure the alterations found in these diseases. We do have, however, data on young normal puppy cartilages, and from these data certain tentative conjectures of value may be reasonably made, since we have found from chemical studies on the soft tissues of the dog[2] that such soft tissues are very similar in composition to those of the human being. Fully realizing the limitations of such postulations, we plan to obtain and study the cartilage from normal human subjects as soon as practicable.

In the preceding paper[1] the quantitative amounts and distribution of water, electrolytes, connective tissue, and chondroitin sulfate of the hyaline cartilage of normal puppies were given. The purpose of the present paper is to formulate similar quantitative data on the hyaline cartilages removed from the joint surfaces of poliomyelitis patients at the time of various reconstructive procedures. These data have been interpreted histochemically and are believed to provide a first approximation of: (1) the relative mass of fibrillary material, chondroitin sulfate, and chondrocytes; and (2) their distribution and the distribution of water and electrolytes in the intra- and extracellular compartments.

The electrolyte content of human cartilage has not been studied comprehensively in the past, and no data on the chemical characteristics of cartilage removed from poliomyelitis patients could be found in the literature. The available data on the chemical analyses of cartilage have been summarized in the previous paper.[1]

METHODS AND PROCEDURES

Thirteen cartilage specimens were available for analysis: seven from the foot; one from the ankle; two from the knee; and three from the shoulder. The tissues were removed at the time of operation and placed immediately in a glass-stoppered jar. Usually the specimen consisted of hyaline cartilage and articular cortices from opposing joint surfaces. The reconstructive procedures were arthrodesing operations in the foot ("triple arthrodesis"), ankle, knee, and shoulder. The specimens were removed to the laboratory where the hyaline

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TABLE I. ORIGINAL DATA: ANALYSES OF HYALINE CARTILAGE AND SERUM FROM POLIOMYELITIS PATIENTS

PATIENT ORIGIN OF TISSUE	AGE (YR.:MO.)	DURATION OF PARALYSIS (YR.:MO.)	TISSUE	H_2O (GM.)	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	CA (MEQ.)	MG (MEQ.)	TOTAL N (GM.)	COL-LAGEN N (GM.)	SO_4 (MM)
26 GY	5:5	4:8	Serum	905.0	105.2	135.4				12.27		
Foot			Cartilage	741.5	60.8	186.5	13.44			34.42	23.21	138.0
28 FR	6:	4:8	Serum									
Foot			Cartilage	745.0	53.9	172.9	11.02	36.6	24.0	34.55	23.54	88.0
22 LW	7:	5:2	Serum	908.5	100.6	135.8	4.97			12.76		
Foot			Cartilage	710.5	108.8					42.20	31.50	88.5
10 DE	7:	0:8	Serum	916.1	103.2	138.2	5.15			10.92		
Foot			Cartilage	732.8		169.1	11.67	23.9	9.5	37.00	26.42	62.8
9 LJ	10:	8:10	Serum									
Foot			Cartilage	719.5	69.0	178.7	14.53			37.40	27.48	62.8
33 BM	13:4	12:10	Serum	920.1	98.9	133.6	4.57			10.38		
Knee			Cartilage	767.2	56.2	139.6	9.26	43.1	10.3	32.71	23.25	37.4
30 JM	14:	1:11	Serum	908.3	99.1	133.4						
Foot			Cartilage	680.3	70.0					45.88	33.53	42.8
5 TH	14:	12:	Serum	908.9	100.2					10.42		
Shoulder			Cartilage	697.0		176.6	16.70	58.0	8.2	43.55	31.16	52.7
34 JM	16:	1:10	Serum	917.6	100.6	136.8	4.57			11.70		
Shoulder			Cartilage	748.6	69.3	163.4	12.70	39.6	22.9	35.10	25.88	43.0
25 NI	17:	15:	Serum	922.2	104.7	135.2	5.32			10.70		
Ankle			Cartilage	696.5						41.27	31.13	67.7
31 AK	17:	3:8	Serum	905.4	99.2	135.4	4.69			12.66		
Shoulder			Cartilage	743.8	76.1	161.2	12.07	18.1	23.3	39.15	29.00	25.4
X7 YE	18:	8:4	Serum	905.9	103.8	135.0				11.24		
Foot			Cartilage	692.0						46.19	33.57	78.8
8 DS	19:	16:10	Serum	911.8	102.2	139.0				11.62		
Knee			Cartilage	730.9	83.9	177.1	13.18	32.7	7.2	38.95	27.40	49.5

The units are expressed per kilogram of tissue.

cartilage was trimmed from the bone with a scalpel. Extreme care was taken to avoid cutting into the zone of provisional calcification or into the articular cortex. The cartilage fragments were placed immediately in a previously weighed glass-stoppered weighing bottle, and the weight of the specimen was determined. All of the procedures were carried out as quickly as possible to avert water loss. The cartilage was then dried to constant weight in a 100° C. thermostatically controlled oven, and the water content of the specimen was determined after reweighing. The dried cartilage was next transferred quantitatively to a special apparatus and crushed by a technique described in a former paper.[1] The specimen was again placed in the 100° C. oven for three or four hours, and then stored in a desiccator over activated aluminum oxide. For all determinations, aliquots were removed from the bottle after thorough mixing, and weighed as quickly as possible to minimize absorption of water by the dried specimen.

The chemical methods used in the analyses were the same as those employed in our previous work.[1] The determinations made upon the pulverized cartilage were the following: chloride, sodium, potassium, calcium, magnesium, total nitrogen, collagen nitrogen, and sulfate. Insufficient amounts of cartilage in some of the specimens limited the number of determinations that could be performed. Since the calcium and magnesium determinations required the largest amount of the specimen, they were the most frequently omitted.

Serum determinations were made on all except two of the thirteen patients. Unfortunately, not all of the serum specimens were obtained at the time of surgery. However, enough serum specimens were obtained at the time of operation to show that there was no significant difference in the serums obtained then and those obtained later. The serum nitrogen determinations showed the greatest variation, but these were not important in this study. Since the chloride determinations were so consistent, a reasonably accurate estimation of the chloride value in the serum of the two unavailable patients could be made.

RESULTS

Values for Constituents of Serum and Cartilage.—Tables I and II present the original analytical data on the cartilage and serum of the thirteen patients. The patients are arranged by chronological age at the time of the surgical procedures. The origin of the tissue and the duration of the paralysis is given for each. In Table I, the results are expressed in units per kilo of wet fresh tissue; in Table II, the results are given in units per 100 Gm. of dried tissue solids. Since the water content of the thirteen cartilages varied between 68 and 76 per cent, it is possible that some of the water may have been lost during the

TABLE II. ANALYSES OF HYALINE CARTILAGE FROM POLIOMYELITIS PATIENTS

PATIENT	ORIGIN OF TISSUE	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	CA (MEQ.)	MG (MEQ.)	TOTAL N (GM.)	COLLAGEN N (GM.)	SO_4 (MM)
26 GY	Foot	23.55	72.2	5.20			13.32	8.98	53.4
28 FR	Foot	21.16	67.8	4.32	14.35	9.43	13.55	9.23	34.5
22 LW	Foot	37.6					14.58	10.88	30.6
10 DE	Foot		63.3	4.37	8.94	3.56	13.85	9.89	23.5
9 LJ	Foot	24.60	63.7	5.18			13.33	9.80	22.4
33 BM	Knee	24.18	60.0	3.98	18.55	4.44	14.06	9.99	16.1
30 JM	Foot	21.90					14.35	10.49	13.4
5 TH	Shoulder		58.3	5.51	19.14	2.70	14.37	10.28	17.6
34 JM	Shoulder	27.58	65.0	5.05	15.77	9.10	13.97	10.29	17.1
25 NI	Ankle						13.60	10.26	22.3
31 AK	Shoulder	29.70	62.9	4.71	7.07	9.08	15.28	11.32	9.1
X7 YE	Foot						14.99	10.90	25.1
8 DS	Knee	31.20	65.8	4.90	12.17	2.68	14.48	10.18	18.4

The values are expressed in units per 100 Gm. of dried cartilage solid.

period of removal of the specimen. It was not possible for us to obtain the specimen and to remove the cartilage under perfectly uniform conditions. Therefore, the difference in water content may be real, or it may be the result of unavoidable delay in processing the material. In view of this possible source of error, the data are additionally expressed in units per 100 Gm. of dried cartilage solids (Table II).

Derived Data.—Using the original data of Tables I and II, we have calculated the histochemical characterization of these cartilages per 100 Gm. of cartilage solids and per kilogram of fresh cartilage. Several preliminary statements are required to justify these calculations. Histologically, cartilage is known to consist of cells, extracellular fluid, connective tissue fibers, and chondroitin sulfate. If this tissue is similar to other tissues of the body, and it is reasonable to suppose that it is, the cartilage may be considered to consist of cells and extracellular solids surrounded by extracellular fluid whose composition is deduced from analyses of serum. Under this premise, the pattern of cartilage may be divided into intra- and extracellular compartments: the intracellular compartment consisting of the cells and their associated water, the extracellular compartment consisting of a connective tissue phase containing some of the extracellular fluid, a chondroitin sulfate phase, and the remaining extracellular water (ultrafiltrate) in the interstices. This extracellular compartment is equivalent to the "ground substance," terminology used by histologists.[3]

Fundamental to these calculations is the assumption that the connective tissue of cartilage corresponds to all the other connective tissues of the body, whether loose or dense. Such an assumption is not without support, for Manery and associates[4] showed experimentally the similarity between loose and dense connective tissue in the electrolyte concentrations, and concluded that the electrolyte patterns of all connective tissues resemble each other closely. Partridge[5] has stated that the ground substance of cartilage is similar to that present in loose connective tissue. From such evidence we have judged that the connective tissue of cartilage resembles histochemically that of tendon which is probably the most dense and uniform connective tissue of the body containing as it does the most fibrils, the least ground substance, and the least cellular diversity. In a previous paper,[6] the electrolyte pattern of dog tendon has been determined and the following values were established: 100 Gm. of tendon solids (connective tissue solids) were found to be associated with 157 Gm. of water, 17.2 Gm. of total nitrogen, 15.8 Gm. of collagen nitrogen, 20.65 meq. of chloride, 20.20 meq. of sodium, and 3.4 meq. of potassium.[4] Thus, collagen nitrogen is 92 per cent of the total connective tissue nitrogen. Recently, we have been fortunate to be able to study a number of human tendons from amputated extremities and have found them to be quite similar in composition to dog tendons.

With the conviction that the collagen nitrogen of cartilage exemplifies the total connective tissue of cartilage, we have calculated the amount of connective tissue in 100 Gm. of cartilage solids and, in addition, the amount of chloride, sodium, and potassium associated with these amounts of connective tissue. Since collagen nitrogen is 92 per cent of the total connective tissue nitrogen, the

TABLE III. ESTIMATED WEIGHT OF CONNECTIVE TISSUE SOLIDS IN ALL CARTILAGES

PATIENT	CARTILAGE	COLLEGAN N (GM.)	CONNECTIVE TISSUE N (GM.)	ΔN (GM.)	CONNECTIVE TISSUE SOLIDS (GM.)	Δ SOLIDS (GM.)
26 GY	Foot	8.98	9.76	3.56	56.7	43.3
28 FR	Foot	9.23	10.02	3.53	58.4	41.6
22 LW	Foot	10.82	11.76	2.82	68.4	31.6
10 DE	Foot	9.89	10.75	3.10	62.5	37.5
9 LJ	Foot	9.80	10.65	2.68	61.9	38.1
33 BM	Knee	9.99	10.86	3.20	63.2	36.8
30 JM	Foot	10.49	11.41	2.94	66.3	33.7
5 TH	Shoulder	10.28	11.17	3.20	64.9	35.1
34 JM	Shoulder	10.29	11.18	2.79	65.0	35.0
25 NI	Ankle	10.26	11.16	2.44	64.9	35.1
31 AK	Shoulder	11.32	12.30	2.98	71.5	28.5
X7 YE	Foot	10.90	11.84	3.15	68.9	31.1
8 DS	Knee	10.18	11.07	3.41	64.3	35.6

The values are given in units per 100 Gm. of solids.

△ = Total cartilage amount minus fiber tissue amount.

ΔN = Nitrogen not associated with the connective tissue: = Total nitrogen – connective tissue nitrogen.

△ Solids = 100 – connective tissue solids.

amount of connective tissue nitrogen can be readily estimated. The amount of connective tissue solids which would contain this amount of connective tissue nitrogen is then determinable. For example: if 17.2 Gm. of total connective tissue nitrogen represents 100 Gm. of connective tissue solids, it is obvious that we can calculate the weight of connective tissue solids represented by any known amount of connective tissue nitrogen. In Table III the estimated connective tissue solids in the cartilages of all patients are presented in units per 100 Gm. of cartilage solids. The estimated amounts of chloride, sodium, and potassium associated with this calculated amount of connective tissue solids are presented in Table IV. The amounts of the constituents in excess of those associated with the connective tissue solids are labeled with the figure △. The △ values are of importance, for they represent the concentration of the constituents in the other

TABLE IV. THE CALCULATED AMOUNTS OF CHLORIDE, SODIUM AND POTASSIUM ASSOCIATED WITH THE CONNECTIVE TISSUE SOLIDS IN ALL CARTILAGES

PATIENT	CARTILAGE	CONNECTIVE TISSUE SOLIDS (GM.)	Δ SOLIDS (GM.)	CONNECTIVE TISSUE CL (MEQ.)	Δ CL (MEQ.)	CONNECTIVE TISSUE NA (MEQ.)	Δ NA (MEQ.)	CONNECTIVE TISSUE K (MEQ.)	Δ K (MEQ.)
26 GY	Foot	56.4	43.3	11.72	11.83	11.46	60.7	1.93	3.27
28 FR	Foot	58.4	41.6	12.06	9.10	11.80	56.0	1.99	2.33
22 LW	Foot	68.4	31.6	14.13	23.47	13.82		2.32	
10 DE	Foot	62.5	37.5	12.91		12.62	50.7	2.12	2.24
9 LJ	Foot	61.9	38.1	12.78	11.82	12.51	51.2	2.11	3.08
33 BM	Knee	63.2	36.8	13.05	11.13	12.77	47.2	2.15	1.83
30 JM	Foot	66.3	33.7	13.69	8.21	13.39		2.25	
5 TH	Shoulder	64.9	35.1	13.41		13.11	45.2	2.21	3.30
31 JM	Shoulder	65.0	35.0	13.42	14.16	13.13	51.9	2.21	2.84
25 NI	Ankle	64.9	35.1	13.41		13.11		2.21	
31 AK	Shoulder	71.5	28.5	14.76	14.94	14.44	48.5	2.43	2.28
X7 YE	Foot	68.9	31.1	14.23		13.92		2.34	
8 DS	Knee	64.4	35.6	13.29	17.91	13.00	52.8	2.19	2.71

The values are given in units per 100 Gm. of solids.

△ = Total cartilage concentration minus estimated connective tissue concentration.

compartments of the cartilages. The △ nitrogen and potassium, therefore, should represent the quantity of these constituents in the chondrocytes; the △ chloride the ultrafiltrate volume; and the △ sodium primarily the amount of sodium associated with the chondroitin sulfate.

It is now possible to partition the total solids of cartilage into the intra- and extracellular solids per 100 Gm. of dried cartilage, and then to convert and express these values per kilogram of cartilage tissue. As previously stated, the extracellular phase of the dried cartilage contains the connective tissue solids (fiber solids), chondroitin sulfate, and the associated sodium. In Table V, these values are given, together with the total amount of intra- and extracellular solids in 100 Gm. of dried cartilage solids. If the △ nitrogen represents the quantity of nitrogen within the chondrocytes, the weight of the protein equivalent to the △ nitrogen should approximate the weight of the cellular solids. From Table V it is apparent that the weights of the cellular solids thus calculated are almost equivalent to the weights obtained by difference, that is by subtracting the weight of the estimated extracellular solids from the 100 Gm. of total solids.

In Table VI the data for the estimations of the total weights of the extracellular and intracellular solids per kilogram of fresh cartilage tissue are given. A further characterization of the hyaline cartilages of the poliomyelitis patients per kilogram of fresh tissue is given in Table VII. These derived data partition the components into the two main phases of the tissue. Incomplete data made it necessary to include only eight of the thirteen patients in these computations.

This division of the component parts of fresh cartilage is derived from the original data and from the characterization of the constituents in 100 Gm. of

TABLE V. HISTOCHEMICAL DATA FOR 100 GRAMS OF HYALINE CARTILAGE SOLIDS

PATIENT	CARTILAGE	$(F)_s$ (GM.)	CSA_s (GM.)	WEIGHT ΔNA_s (GM.)	$(E)_s$ (GM.)	$(C)_s$ (GM.)	ΔN (GM.)	$(C)_s$ CALCULATED FROM ΔN (GM.)
26 GY	Foot	56.7	25.0	1.40	83	17	3.6	22
28 FR	Foot	58.4	16.1	1.28	76	24	3.5	22
22 LW	Foot	68.4	14.3	1.19*	84	16	2.8	17
10 DE	Foot	62.5	11.0	1.16	75	25	3.1	19
9 LJ	Foot	61.9	10.5	1.18	74	26	2.7	17
33 BM	Knee	63.2	7.5	1.09	72	28	3.2	20
30 JM	Foot	66.3	6.3	1.19*	74	26	2.9	18
5 TH	Shoulder	64.9	8.2	1.04	74	26	3.2	20
34 JM	Shoulder	65.0	17.1	1.19	83	17	2.8	17
25 NI	Ankle	64.9	10.4	1.19*	77	23	2.4	15
31 AK	Shoulder	71.5	4.6	1.11	77	23	3.0	19
X7 YE	Foot	68.9	12.0	1.19*	82	18	3.1	20
8 DS	Knee	64.4	8.6	1.22	74	26	3.4	21

Estimated weight of extracellular $(E)_s$ and intracellular $(C)_s$ solids in 100 Gm. of cartilage solids.

The values are given in units per 100 Gm. of total solids.

*Assumed weight of ΔNa_s (insufficient material for analysis) based upon the average of the other determinations.

$(F)_s$ = Connective tissue solids (Table III). Hereafter called fiber solids.

CSA_s = Chondroitin sulfate solid (calculated from Levene's molecular weight).

ΔNa_s = Weight of ΔNa (Table IV) in grams.

$(E)_s$ = Extracellular solids. $(E)_s = (F)_s + CSA_s + \Delta Na_s$.

$(C)_s$ = Intracellular solids. $(C)_s = 100$ Gm. $- (E)_s$.

ΔN = Nonconnective tissue nitrogen (Table III).

TABLE VI. PHASE MASS DATA FOR ONE KILOGRAM OF HYALINE CARTILAGE. (DERIVED SOLIDS)

PATIENT	CARTILAGE	TOTAL WATER (GM.)	TOTAL SOLIDS (GM.)	$(F)_S$ (GM.)	CSA (GM.)	WEIGHT ΔNA (GM.)	$(E)_S$ (GM.)	$(C)_S$ (GM.)
26 GY	Foot	741	259	146.6	64.6	3.61	214.8	43.7
28 FR	Foot	745	255	148.9	41.1	3.28	193.3	61.7
22 LW	Foot	710	290	198.0	41.5	3.45*	242.9	47.1
10 DE	Foot	733	267	167.0	19.4	3.11	189.5	77.7
9 LJ	Foot	719	281	173.6	29.4	3.30	206.3	74.2
33 BM	Knee	767	233	147.1	17.5	2.53	167.1	65.7
30 JM	Foot	680	320	211.8	20.1	3.81*	235.7	84.3
5 TH	Shoulder	697	303	196.7	24.8	3.15	224.7	78.3
34 JM	Shoulder	749	251	163.4	43.0	3.00	209.4	42.0
25 NI	Ankle	696	304	196.7	31.7	3.62*	232.0	72.0
31 AK	Shoulder	744	256	183.2	11.9	2.85	198.0	58.2
X7 YE	Foot	692	308	212.1	36.9	3.66*	252.6	55.4
8 DS	Knee	731	269	173.2	22.5	3.27	199.0	70.1

* = Assumed weight △Na. (Table V.)
$(F)_S$ = gram connective tissue solids per kilogram = $(F)_S$ (Table V) × total solids/100.
CSA = gram chondroitin sulfate per kg. = CSA_S (Table V) × total solids/100.
Weight △Na = wgt. $\triangle Na_S$ (Table V) × total solids per 100.
$(E)_S$ = gram solids of extracellular compartment; = $(F)_S$ + CSA + △Na.
$(C)_S$ = gram solid of intracellular compartment; = Total solids—$(E)_S$.

cartilage solids (Table V). With the concentration of the constituents in 100 Gm. of cartilage solids now established, and with the total water and the total solids per kilogram of fresh tissue recorded in Table VI, we are able to estimate the partition of all constituents in the total solid content of one kilogram of fresh cartilage. These computations are shown in detail in Table VII.

The assignment of the fractional parts of the total water into the separate phases is given in Table VII. The water identified with the extracellular phase $(H_2O)_E$ has been considered to consist of the water associated with the connective tissue solids $(H_2O)_F$, plus the water of the ultrafiltrate $(H_2O)_U$. The water identified with the cells, or the intracellular water $(H_2O)_C$, is thus the water by difference, that is the total water value minus the extracellular water value. The proposed conception that the △ chloride represents the ultrafiltrate space was utilized to calculate the ultrafiltrate phase. The volume of this space was deduced from the serum chloride values in Table I, by means of equations presented in former papers.[7]

DISCUSSION

We have endeavored to present histochemical patterns of hyaline cartilages removed from the joint surfaces of patients with the residual paralysis of poliomyelitis. The validity of all of the derived estimations presented here depends upon the correctness of two assumptions, namely: (1) that the connective tissue of hyaline cartilage is like any other connective tissue of the body in water and electrolyte concentration, and (2) that the chloride not associated with connective tissue (△Cl) may be considered to be confined to the extracellular water.

The data from the tissues presented here were considered on the basis of 100 Gm. of dry cartilage solids and on the basis of fresh tissue which was divided into two phases, extracellular and intracellular. Fig. 1 shows graphically the

TABLE VII. PHASE MASS DATA FOR ONE KILOGRAM OF HYALINE CARTILAGE. (DERIVED WATER)

PATIENT	CARTILAGE	$(F)_S$ (GM.)	$(H_2O)_F$ (GM.)	Δ CL (MEQ.)	CL_U (MEQ.)	$(H_2O)_U$ (GM.)	$(H_2O)_E$ (GM.)	$(E)_S$ (GM.)	$(E)_T$ (GM.)	$(C)_T$ (GM.)	$(H_2O)_C$ (GM.)	$(C)_S$ (GM.)
26 GY	Foot	146.6	230.1	30.60	120.8	253	483	215	698	302	258	44
28 FR	Foot	148.9	233.7	23.19	116.4*	199	433	193	626	374	312	62
9 LJ	Foot	173.6	272.6	33.15	116.4*	287	560	206	767	233	159	74
33 BM	Knee	147.1	231.0	25.83	111.7	289	520	167	687	313	247	66
30 JM	Foot	211.8	332.5	26.23	113.4	231	564	236	800	200	116	84
34 JM	Shoulder	163.4	256.5	35.60	114.2	312	568	209	778	222	180	42
31 AK	Shoulder	183.2	287.7	38.30	114.0	336	624	198	822	178	120	58
8 DS	Knee	173.2	272.0	48.25	118.8	406	678	199	877	123	53	70

*Assumed meq. of Cl_U based upon the average of all other determinations in the series.

$(F)_S$ = gram connective tissue solids per kilogram (Table VI).

$(H_2O)_F$ = gram water associated with $(F)_S$.

$\Delta Cl = \Delta Cl_S$ (Table IV) $\times$ total solids/100.

Cl_U = meq. Cl per kilogram serum water $\times$ 0.0099/0.0095.

$(H_2O)_U = \Delta Cl/(Cl)_U \times 1{,}000$.

$(H_2O)_E$ = gram of extracellular water per kilogram cartilage = $(H_2O)_U + (H_2O)_F$.

$(E)_S$ = gram of solids of extracellular compartment (Table VI).

$(E)_T$ = gram of extracellular phase per kilogram cartilage = $(H_2O)_E + (E)_S$.

$(C)_T$ = gram of intracellular phase per kilogram cartilage = $(H_2O)_C + (C)_S$.

$(H_2O)_C$ = gram of intracellular water = (Total water content − Extracellular water $[H_2O]_E$).

division of the 100 Gm. of solids into connective tissue solids, chondroitin sulfate solids, and the cell solids; Fig. 3, the phase mass data for one kilogram of fresh cartilage.

In soft tissues such as muscle and liver, the total solids represent primarily the proteins of the tissue cells (intracellular solids).[8, 9] Conversely, in cartilage the total solids represent mostly the solids of the extracellular phase (ground substance) and the small protein fraction of the chondrocytes. From Fig. 1, it seems reasonable to ascribe approximately 75 per cent of the cartilage solids of these patients to the extracellular solids and 25 per cent to the cellular solids. This generalization does not vary much from the values obtained on articular

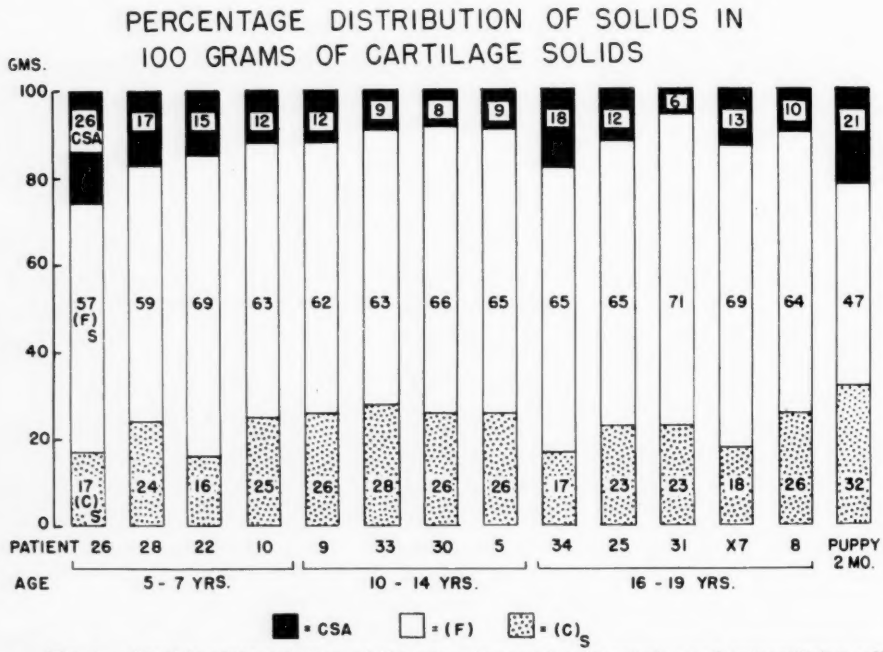

Fig. 1.—Graphic representation of the percentages of extracellular and intracellular solids in 100 Gm. of total cartilage solids. The percentage of the chondroitin sulfate solids, illustrated by the black areas, plus the percentage of the connective tissue solids, illustrated by the unshaded areas, represent the extracellular solids. The percentage of the cell solids is represented by the dotted areas.

cartilages removed from puppies, of which 70 per cent of the cartilage solids was found to be extracellular and 30 per cent was cellular. Great differences are noted, however, when the extracellular solids are considered from the standpoint of their separate parts, namely, connective tissue solids and chondroitin sulfate solids. Consequently, considering only the extracellular solids (ground substance), the percentages of the connective tissue solids and the chondroitin sulfate solids per 100 Gm. of extracellular solids have been calculated, and these values are shown graphically in Fig. 2. Young puppy articular cartilage is included for reference. Of the extracellular solids in these patients the connective tissue solids are a much larger component than the chondroitin sulfate solids. Furthermore the connective tissue solids varied inversely with the chondroitin

sulfate solids. More experimentation needs to be carried out before it can be decided definitely whether these variances are significant: whether they are the result of the dysfunction from the disease residuals, or the effect of age, or of disuse atrophy.

Observations on the group of patients rule out somewhat the age effects. For example, if patients 26 and 28 are conceded to have normal chondroitin sulfate distribution for their age group (5 to 7 years), then patients 22 and 10 who belong in the same age group should have higher values than were found. Patients 9, 33, 30, and 5 (10 to 14 years) have a lower chondroitin sulfate percentage in their extracellular solids than patient 34, who was 16 years of age.

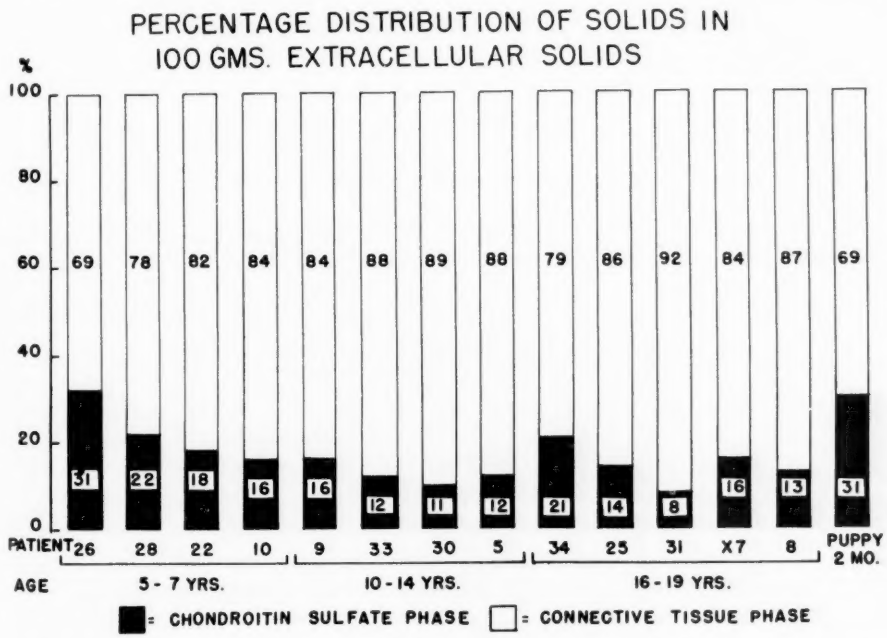
PERCENTAGE DISTRIBUTION OF SOLIDS IN
100 GMS. EXTRACELLULAR SOLIDS
%
100
80
60
40
20
0
69 78 82 84 84 88 89 88 79 86 92 84 87 69
31 22 18 16 16 12 11 12 21 14 8 16 13 31
PATIENT 26 28 22 10 9 33 30 5 34 25 31 X7 8 PUPPY 2 MO.
AGE
5 - 7 YRS.
10 - 14 YRS.
16 - 19 YRS.
= CHONDROITIN SULFATE PHASE
= CONNECTIVE TISSUE PHASE

Fig. 2.—Graphic representation of the percentages of connective tissue solids and chondroitin sulfate solids in 100 Gm. of extracellular solids. The percentage of the connective tissue solids is illustrated by the clear areas, and the percentage of the chondroitin sulfate solids by the black areas.

This suggests the possibility that the effect of age is not the sole controlling factor. The duration of paralysis, another variable, may also be excluded as a single controlling factor. Patient 31 who has the lowest percentage of chondroitin sulfate and the highest percentage of connective tissue solids had been paralyzed for approximately four years—about one-third as long as patient 5 who has twice as much chondroitin sulfate and less connective tissue solids. It seems likely, therefore, that these findings must be attributed to a number of variances occurring simultaneously, or to the manifold effects of the disease. The extent of paralysis may be a factor, but this is extremely difficult to appraise clinically. Disuse may also be a factor, but this is even more difficult to evaluate because it would involve assessment of the paralysis about the joint from which the cartilage was removed, assessment of the associated paralysis of the trunk

and other extremities, and an estimation of the absolute activity of the patient at the time of the operation. It is noteworthy that in these cartilages, contrary to the speculations of Hass,[10] there was no pigmentation present, although the chondroitin sulfate values were low. In his work on aging costal cartilages, Hass noted pigmentation and speculated that the pigments may be a degradation product of the chondroitin sulfate, because the pigmentation appeared as the chondroitin sulfate decreased in quantity.

The assembled data for the masses of the extra- and intracellular phases for one kilogram of fresh cartilage are graphically shown in Fig. 3 for eight of the thirteen patients studied. Representatives from each of the three age groups are present. Puppy articular cartilage is again included for reference.

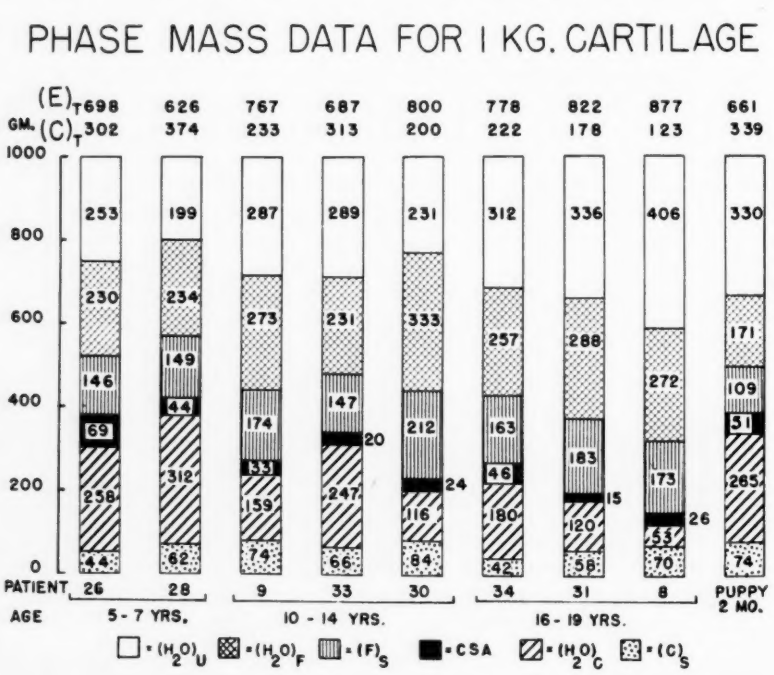

Fig. 3.—Phase mass data for one kilogram of hyaline cartilage. Graphic representation of the extracellular phase consisting of (1) the ultrafiltrate water illustrated by the clear areas, (2) the connective tissue phase consisting of the connective tissue solids illustrated by the perpendicular cross hatched areas and the associated water illustrated by the double cross hatched areas, and (3) the chondroitin sulfate phase illustrated by the black area. The intracellular phase consisting of the intracellular water illustrated by the single diagonally hatched areas, and the intracellular solids illustrated by the dotted areas.

We have identified the extracellular phase with the chondroitin sulfate solids, the connective tissue solids with their associated water and the serum ultrafiltrate.[5] The intracellular phase is identified with the chondrocytes and their associated water. This conception of the extracellular phase of cartilage should prove of importance in studying any diseased or abnormal condition in cartilage. The concurrent connective tissue and chondroitin sulfate alterations in these conditions of paralyses are further illustrated. It will be noted that if the weight of the connective tissue phase, which includes the connective tissue solids

and their associated water, is considered, there is only moderate variation. For example, in the 5- to 7-year age group, patient 26 has a connective tissue phase weight of 376 Gm., and patient 28 has a phase weight of 383 Gm.; patients 9, 33, and 30 (10 to 14 years) have a connective tissue phase weight of 446, 379, and 545 Gm., respectively; patients 31, 34, and 8 (16 to 19 years) have connective tissue phase weights of 471, 419, and 445 Gm., respectively. If these values for the weight of the extracellular connective tissue phase prove to be large in comparison with normal human beings, the alteration probably is the result of the effects of the disease, since age and duration of paralysis have been partially excluded. Unfortunately, we do not have at present normal human cartilages for comparison.

The relative mass of the solids of the chondrocytes per kilogram of cartilage varies between 4.2 per cent and 8.4 per cent. If the mass of the solids of the chondrocytes represent the weight of the proteins of these cells, this finding confirms that the number of cells in cartilage tissue per unit weight is small. Normally, the quantity of potassium in the cells is more or less proportional to the mass of protein. Therefore, since the mass of protein in these cartilages is low, the potassium values should also be low. From Table IV it will be noted that the potassium which has been assigned to the cells, and labeled $\triangle$ K, is low. In fact, when the $\triangle$ K is calculated for one kilogram of chondrocytes, there is found a large deficit of intracellular potassium in all of the patients. $(\text{K/Kg. cells} = \triangle \text{ K} \times \frac{(\text{solids per kilogram cartilage})}{100} \times \frac{1000}{(\text{C})_{\text{T}}})$. Considering the articular puppy cartilage values as normal tissue values, the calculated potassium concentration per kilogram of chondrocytes was found to have an average value of 145 meq. In the seven patients in which we have complete data, numbers 26, 28, 9, 33, 34, 31, and 8, the calculated potassium values per kilogram of cells were, respectively, as follows: 28, 16, 37, 14, 32, 33, and 43 meq. These extremely low potassium values indicate that abnormal amounts of sodium must have entered these cells to replace the huge deficit of potassium in order to have ionic equilibrium. The potassium freed by the cells must have been the result of a disturbed equilibrium in the cells. That some of the sodium entered the cells is experimentally suggestive, since the values for the $\triangle$ sodium in all of the patients were considerable, in lieu of the low chondroitin sulfate values; sodium must have gone from the extracellular compartment into the cells. Darrow[11] has reviewed conditions in which sodium entered other tissue cells when there was a deficit of potassium. Potassium deficits can produce serious conditions in tissues, for example Ferrebee and associates[12] have shown that the deficiency of body potassium produces necrosis of the heart muscle in dogs.

The water of the chondrocytes, or the cellular water as illustrated in the diagram, has been considered in terms of percentage of the intracellular phase. For puppy articular cartilages, the percentage of water per kilogram of cells was found to have an average value of 78 per cent. In these human cartilages the calculated values of the percentages of intracellular water were as follows: in patients 26 and 28 the percentage of intracellular water in each was 85 per

cent; in the next age group, patients 9, 33, and 30 had percentages of 68, 79, and 53, respectively; and in the oldest group, patients 31, 34, and 8, the percentages were 67, 81, and 43, respectively. It is obvious, therefore, that patients 9, 30, 31, and 8 had low percentages of cell water, or a state of dehydration of the cells existed. These same patients also had low chondroitin sulfate values. The fact that dehydrated cells do not metabolize or function physiologically offers the provocative suggestion that the existing state of dehydration limited the formation of chondroitin sulfate in these cartilages.

SUMMARY

Chemical data are presented for the values of hyaline cartilages removed from the joint surfaces of poliomyelitis patients at the time of various reconstructive procedures. These data are utilized to describe (a) the percentages of extracellular and intracellular solids in 100 Gm. of the total cartilage solids, (b) the percentages of connective tissue solids and chondroitin sulfate solids in 100 Gm. of extracellular solids, and (c) the histochemical characterization of the extra- and intracellular phases per kilogram of fresh cartilage.

Discussion is given in reference to whether the variances found in the percentages of the extracellular solids (connective tissue solids and chondroitin sulfate) are the result of the dysfunction from the disease, or the effect of age, or of disuse atrophy. Commentary is also made on the possibility that the derived phases of cartilage may in the future prove serviceable in studying diseased or abnormal conditions in cartilages.

We wish to express our appreciation to Dr. C. Howard Hatcher and Dr. Mary S. Sherman for making these tissues available to us for study.

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LABORATORY METHODS

COMPARATIVE EFFECTIVENESS OF DIRECT BLOOD AGAR AND MODIFIED PIKE'S BROTH FOR DEMONSTRATING BETA-HEMOLYTIC STREPTOCOCCI OF DIFFERENT SEROLOGICAL GROUPS

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IT WAS reported by Pike (1944) that the isolation of beta-hemolytic streptococci from cultures of the nose and throat could be improved measurably by incubating the specimen swab in a selective inhibitory broth prior to seeding the blood agar plate. The medium is known by that author's name, and its composition was described (Pike, 1944) as follows: "Beef heart infusion broth containing 1 per cent tryptose, 0.02 per cent glucose, and 5 per cent rabbit blood was tubed in 2 ml. amounts. On the day the medium was used, to each tube was added 0.15 ml. of an autoclaved aqueous solution of sodium azide, 1:1000, and 0.1 ml. of an autoclaved aqueous solution of crystal violet, 1:25,000."

From the initiation of the intramural streptococcal control program at the Wieting-Johnson Hospital for Rheumatic Diseases, it has been our custom to seed a sheep's blood agar plate directly from the cotton swab and then to repeat this procedure after incubating the same swab overnight in a tube of modified Pike's broth. It is the purpose of this report to present the comparative data which have been collected from 4,176 such nose and throat cultures. Beta-hemolytic streptococci were found in 446 (16 per cent) of these specimens. Additional information elicited from studies with modified Pike's broth made from different dehydrated bases also will be presented.

MATERIALS AND METHODS

Pike's Broth.—Except where indicated below, the broth utilized for this study was made as follows: Trypticase soy broth was tubed in 2 ml. amounts and stored in the refrigerator until needed, at which time the following were added to each tube.

0.15 ml. sodium azide 1:1000 (autoclaved)
0.10 ml. crystal violet 1:25,000 (autoclaved)
0.10 ml. defibrinated sheep blood

This medium differs from that originally described by Pike in that trypticase soy broth forms the base, sheep replaces rabbit blood, and both this and the inhibitory agents are added to the broth immediately prior to use.

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Blood Agar Plates.—Defibrinated sheep's blood in 5 per cent concentration in a base of trypticase soy agar.

Cultures.—A sterile cotton swab was wiped across the tonsillar areas and the posterior pharynx (or the anterior nares) and minutes later used to inoculate a portion of a blood agar plate. The swab was then placed in a tube (150 by 16 mm.) containing the freshly prepared modified Pike's broth. The original plate inoculum was then streaked out with a bacteriological needle. The swab and broth were incubated at 37.5° C. for sixteen to eighteen hours, at which time another blood agar plate was inoculated from the swab. All plates were read at twenty-four and forty-eight hours, and single colonies of beta-hemolytic streptococci were fished and streaked on fresh plates for purification preparatory to serological identification.

Serological.—Pure cultures were grouped and typed* by the capillary tube method (Fuller, 1938; Maxted, 1948; and Swift and associates, 1943).

RESULTS

During the first few weeks of the culture program, it was noted with some surprise that no streptococci were being isolated from swabs given preliminary incubation in the inhibitory medium, whereas positive cultures were being detected on the direct blood agar plates. The broth which we were using at that time was being made from a tryptose phosphate base. Since the medium as originally described (Pike, 1944) had had a beef heart infusion base, it was decided to compare broths prepared from these two bases. Accordingly, twenty-five swabs were obtained in duplicate from various individuals on different days. The two swabs procured from each patient were numbered and placed in the two broths in alternate sequence. None of the twenty-five swabs incubated in the tryptose-phospate broth Pike's yielded any beta-hemolytic streptococci, whereas 9 (36 per cent) of the twenty-five streaked from the fresh infusion† broth base medium were positive.

A similar series (twenty-six patients) was then made in which fresh infusion† and trypticase soy broths were compared as the bases. Six (23 per cent) were positive in each instance. In both groups there was one specimen which was positive from one broth and not from the other. Because of these findings, trypticase soy broth was adopted as the standard base for modified Pike's medium in this laboratory and was utilized for the 4,176 cultures which form the body of this report.

The gross differences in the results obtained by direct plating on sheep's blood agar and after preliminary incubation in the modified Pike's broth are

TABLE I. COMPARATIVE EFFECTIVENESS OF DIRECT BLOOD AGAR AND MODIFIED PIKE'S BROTH IN DETECTING 446 STRAINS OF BETA-HEMOLYTIC STREPTOCOCCI

STREPTOCOCCI ISOLATED FROM	NUMBER	PER CENT
Blood agar plates only	118	27
Pike's only	186	42
Both	142	32
Blood agar plate total	260	58
Pike's total	328	74

*We are indebted to Dr. Elaine L. Updyke and the Communicable Disease Center, U.S.P.H.S., Chamblee, Ga., for the streptococcal grouping and typing sera.

†The fresh infusion broth was obtained through the courtesy of Dr. Orren D. Chapman.

summarized in Table I. While 118 (27 per cent) beta-hemolytic streptococcal strains were isolated only from blood agar, 186 (42 per cent) came only from Pike's and 142 (32 per cent) were found in both media. Of the 446 positive cultures, 260 (58 per cent) grew on the sheep's blood agar plates while 328 (74 per cent) were obtained after preliminary growth in the broth. Thus, an additional 68 (16 per cent) strains were isolated as the result of using the modified Pike's broth. The reasons for this will become apparent below.

The incidences of streptococci of different serological groups were as follows: Group A, 103 strains (23 per cent of the total); Group B, 36 strains (8 per cent); Group C, 77 strains (17 per cent); Group F, 98 strains (22 per cent); and Group G, 132 strains (30 per cent).

The data summarized in Tables II and III demonstrate that the results obtained by the two methods depend to a considerable extent upon the serological groups encountered. In Group A, of primary importance to those interested in rheumatic fever, 55 (53 per cent) strains were isolated by both methods, 21 (20 per cent) from blood agar plates only and 27 (26 per cent) from the modified Pike's only. Thus, of the 103 Group A strains, 75 (73 per cent) were detected on direct blood agar plates while 82 (79 per cent) came from modified Pike's broth.

TABLE II. EFFECTIVENESS OF DIRECT BLOOD AGAR PLATE AND MODIFIED PIKE'S BROTH FOR ISOLATION OF BETA-HEMOLYTIC STREPTOCOCCI OF DIFFERENT SEROLOGICAL GROUPS

STREPTOCOCCAL GROUP	BAP ONLY		PIKE'S ONLY		BOTH		TOTALS	
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
A	21	20	27	26	55	53	103	100
B	0	0	23	64	13	36	36	100
C	56	73	7	9	14	18	77	100
F	32	33	55	56	11	11	98	100
G	9	7	74	56	49	37	132	100
Totals	118	27	186	42	142	32	446	100

The data on Group B are somewhat different since 23 strains (64 per cent) were isolated from the broth, none from plates alone and 13 (46 per cent) from both media. In this group all 36 strains (100 per cent) were detected from the modified Pike's broth and only 13 (36 per cent) on direct sheep's blood agar plates.

TABLE III. RELATIVE EFFICIENCIES OF DIRECT BLOOD AGAR PLATE AND MODIFIED PIKE'S BROTH FOR DETECTING BETA-HEMOLYTIC STREPTOCOCCI OF DIFFERENT SEROLOGICAL GROUPS

STREPTOCOCCAL GROUP	BAP		PIKE'S		TOTALS	
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
A	75	73	82	79	103	100
B	13	36	36	100	36	100
C	70	91	21	27	77	100
F	43	44	66	67	98	100
G	58	44	123	93	132	100
Totals	259	58	328	74	446	100

Group C presents a sharp turn in the other direction since in this case 56 (73 per cent) organisms were isolated on blood agar plates only, 7 (9 per cent) from modified Pike's only, and 14 (18 per cent) from both. In summary, 70 (91 per cent) Group C organisms were detected on blood agar plates and only 21 (27 per cent) from Pike's broth.

In Group F, 32 (33 per cent) organisms were detected only on blood agar plates, 55 (56 per cent) only from modified Pike's and 11 (11 per cent) from both media. Consequently, 43 (44 per cent) Group F strains grew out on the blood agar and 66 (67 per cent) from the modified Pike's.

The results in Group G were quite similar to those in Group B. Nine (7 per cent) strains were detected on blood agar plates only, 74 (56 per cent) from the modified Pike's alone, and 49 (37 per cent) were obtained from both media. Thus, 58 (44 per cent) of the G's grew on direct blood agar plates and 123 (93 per cent) were obtained from modified Pike's broth.

TABLE IV. COMPARISON OF RESULTS OBTAINED WITH DIRECT BLOOD AGAR AND MODIFIED PIKE'S BROTH CULTURE METHODS FOR DETECTING DIFFERENT TYPES AMONG 103 GROUP A STREPTOCOCCAL STRAINS

TYPE	NO.	ISOLATED FROM		
		BAP ONLY	PIKE'S ONLY	BOTH
1	13	3	2	8
3	2	0	1	1
4	1	0	0	1
5	10	0	9	1
6	6	2	0	4
12	7	1	0	6
14	3	1	1	1
18	2	2	0	0
22	5	1	2	2
37	5	1	3	1
41	7	1	0	6
42	9	4	2	3
43	11	2	2	7
N. T.*	22	3	5	14
Total	103	21	27	55

*N. T. = Not typable.

Of the 103 Group A strains, 81 were typable and 22 were not specifically identifiable with the available sera.* It will be noted from Table IV that, with the exception of type 5, there seems to be little difference between the effectiveness of the two methods in detecting Group A streptococci of various types. The numbers, admittedly, are small, but it is of interest that nine of the ten type 5 strains were missed on the direct blood agar plates, but were identified from modified Pike's broth. These ten strains were obtained from cultures made at different times from four children who were quartered in two buildings.

DISCUSSION

We are unable at this time to explain the failures with the inhibitory medium made from tryptose phosphate broth. It is evident that its unsuitability could

*Specific antisera for the following types of Group A beta-hemolytic streptococci were available during this study: 1, 2, 3, 4, 5, 6, 12, 13, 14, 15, 17, 18, 19, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 41, 43, 44, 46, and 47.

be the result of either of two mechanisms: the material is deficient nutritionally or, when combined with the other ingredients, the resultant medium is actually inhibitory for beta-hemolytic streptococci. Neither question was investigated further. We discovered at a later date that Pike (1945) had had a similar experience when tryptose phosphate broth was employed in one of his developmental experiments.

There are some differences between the results obtained in our laboratory and those reported in the original study. Pike (1944) not only obtained a marked increase in positive cultures for beta-hemolytic streptococci when the swabs were first incubated in the inhibitory broth, but also noted that streptococcal colonies were present in significantly greater numbers on the plates inoculated from the broth. Our results in this respect were too irregular to be significant. Among those strains which were grouped serologically, Pike found Groups A and C to show the greatest increases. Group B is not mentioned in that report, while the numbers of Groups F and G strains isolated were too small to indicate any preferences.

The data reported by the Commission on Acute Respiratory Diseases (1948) are not entirely comparable with those reported here. The Commission apparently used the broth medium as originally described, but did not examine direct blood agar plates and subcultures from Pike's broth in parallel. The greatest improvement in detecting beta-hemolytic streptococci seemingly as the result of using Pike's broth occurred among their non-typable Group A's and Groups B, C, and G. The typable Group A strains were detected in only slightly increased numbers from the Pike medium.

In a later study (Commission on Acute Respiratory Diseases, 1949) the same group found that the use of Pike's broth doubled the number of demonstrations of beta-hemolytic streptococci from pharyngeal cultures. Since the streptococci which were encountered are not defined serologically, it is not possible to compare their data satisfactorily with those reported herein.

The data presented in this communication indicate that the results obtained from the use of the inhibitory broth are affected importantly by the serological characteristics of the beta-hemolytic streptococci prevalent in the sample. One has to conclude that the serologically separated groups also have metabolic differences that make Pike's medium either favorable or unfavorable for their multiplication. It is possible that this is also true of the individual types which belong to Group A.

SUMMARY

1. Trypticase soy but not tryptose phosphate broth was found to be a good substitute for infusion broth as a base for Pike's medium.

2. Among 4,176 nose and throat cultures, beta-hemolytic streptococci were found 446 times; 260 (58 per cent) grew on sheep's blood agar plates, while 328 (74 per cent) were isolated after preliminary incubation in modified Pike's broth. The inhibitory medium was most useful in the demonstration of Groups B, F, and G. It seemed to interfere with the isolation of Group C strains and, as employed, increased the Group A isolations only slightly.

3. Nine of the ten type 5 (Group A) strains isolated grew only from the inhibitory broth.

The technical assistance of Mrs. Jane Davis during part of this study is gratefully acknowledged.

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DETERMINATION OF FREE, ESTER, AND TOTAL CHOLESTEROL WITHOUT SAPONIFICATION

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IN A study of serum cholesterol in patients receiving insulin coma therapy, frequent analyses were required to demonstrate a definite pattern of variations during the course of treatment. For this, a procedure has been developed that gives a highly accurate cholesterol partition that can be read on a filter type laboratory photocolorimeter. Total and ester cholesterol are determined without saponification and compared with a standard of cholesteryl acetate. The free cholesterol is determined by a modification of the Schoenheimer and Sperry method.[1] The sum of the ester and free cholesterol values is equal to the value obtained for the total cholesterol. This provides a check on the accuracy of each analysis.

In the development of this procedure, several cholesterol partition methods with numerous modifications were examined. The most widely accepted procedure is that of Schoenheimer and Sperry.[1] Smith and Marble[2] adapted this method and the methods described by Bloor,[3] and by Bloor and Knudson[4] to give a method for the determination of total, free, and ester cholesterol. These methods are accurate (however, generally long) and require very careful attention to such factors as time and temperature of saponification and color development. They are particularly tedious if one wishes to determine the three components independently as a means of checking the accuracy of the results.

In order to shorten the time for a total cholesterol determination, Sperry and Brand[5] modified the saponification procedure of Schoenheimer and Sperry[1] to eliminate the precipitation of the hydrolyzed cholesterol and developed the Liebermann and Burchard reaction in chloroform. By applying this reaction directly to the lipid extract of cholesterol and cholesterol esters, Kaye[6] did away with saponification, even though Yasuda[7] had already shown that cholesterol esters give a deeper color than free cholesterol. Taking this fact into account, Saifer and Kammerer[8] compared their modification of the Liebermann and Burchard reaction in a photoelectric colorimeter against a cholesterol standard made up of equal parts of cholesteryl acetate and cholesterol.

Several attempts have been made to supply a correction factor or constants that could be incorporated into the calculation to compensate for the discrepancies between the color produced by pure cholesterol and the mixture of free and various combined forms present in a serum specimen. Recently, Kingsley and Schaffert[9] published a fairly rapid method wherein a correction factor is applied to the ester value which is in turn used to obtain a correction on the value of the total cholesterol.

Sunderman and Razek[10] have made a careful study of the color produced by the Liebermann-Burchard reaction between cholesterol and the acetic anhydride-sulfuric acid reagent. They have shown the stability of the whole absorption color spectrum in rela-

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tion to time, light exposure, and concentration. Their curves indicated that the color has greatest sensitivity in the range of 660 μ; however, it is much more stable, though less sensitive, at 530 μ. They suggest that analyses would be more accurate at this latter wave length if the amount of the aliquot could be increased or if more serum could be used per volume of extracting solvent.

From these and other sources, a method has been developed which is less tedious, which eliminates the need to control the temperature[1, 5] during color development, and which eliminates the necessity of reading to a maximum color intensity.[8, 9] This was done by using the "54" green light filter in place of the "66" generally used. With a filter type instrument, and without individual timing of samples, reproducible results can be obtained. At 540 μ, changes in transmission with wave length are small[10] and variations with temperature and time are diminished. However, as suggested,[10] this portion of the spectrum requires larger amounts of samples on which to develop the color. Therefore, the ratio of serum to extracting solvent was increased to 1 ml. of serum to a total volume of 10 milliliters. From this extract free and ester cholesterol (the latter without saponification) are determined on one aliquot, and total cholesterol is determined without saponification on a second aliquot. The comparison of these aliquots provides an accurate check on each analysis.

METHOD

Reagents and Special Equipment.—(a) Alcohol, ethyl absolute. (b) Alcohol, ethyl 95 per cent. (c) Acetic anhydride-sulfuric acid mixture.[11] In an Erlenmeyer flask containing nine parts of refrigerated acetic anhydride, add one part of refrigerated concentrated sulfuric acid so that it layers out on the bottom of the flask. Mix slowly, stopper, and place in refrigerator to cool. An amount sufficient to take care of unknowns, standards, and blanks is prepared. The reagent should be water clear. When the mixture is removed from the refrigerator for color development, the flask is placed in an ice bath. (d) Acetic acid, glacial A. C. S. (e) Acetic acid, 10 per cent. (f) Benzin—Petroleum ether A. C. S.; boiling point 30° to 60° C. (g) Digitonin. 1 per cent in 50 per cent alcohol.[12, 13] In a 250 ml. beaker containing 1 Gm. of digitonin, add 100 ml. of 50 per cent (by volume) ethyl alcohol. Warm until digitonin dissolves. (h) Ether, purified and dried for fat extraction. (i) Ether-acetone mixture 2:1.[1] (j) Cholesterol stock standard. Dissolve 500 mg. of pure dry cholesterol in 250 ml. of 95 per cent alcohol. One milliliter is equivalent to 2.00 mg. of cholesterol. (k) Cholesterol working standard. Dilute 3.0 ml. of stock standard to 100 ml. with 95 per cent alcohol. One milliliter is equivalent to 0.06 mg. cholesterol. (l) Cholesteryl acetate stock standard. Dissolve 554.3 mg. of pure dry cholesteryl acetate in 250 ml. of 95 per cent alcohol. One milliliter is equivalent to 2.00 mg. of total cholesterol. (m) Cholesteryl acetate working standard. Dilute 10 ml. of stock standard to 100 ml. with 95 per cent alcohol. Two milliliters are equivalent to 0.40 mg. of total cholesterol. (n) Klett Summerson colorimeter. (o) Green filter No. 54. (p) Macro Klett tubes. (q) Micro Klett tubes.

Extraction of Cholesterol From Serum.—Exactly 9.2 ml.* of absolute alcohol are added to 1.0 ml. of clear unhemolyzed serum in a 15 ml. test tube. It is stoppered with a vaccine bottle rubber stopper, shaken vigorously, and placed so that the contents of the tube are just submerged in a 56° C. water bath for ten minutes. At the end of this extraction period, the tube is removed from the water bath, shaken again vigorously, and centrifuged at 2,750 r.p.m. for ten minutes. The supernatant is a clear alcoholic extract from which aliquots are taken for the determination of total, free and ester cholesterol.

*This amount of alcohol and 1.0 ml. serum gives, due to shrinkage, a final volume of 10.0 ml.

Total Cholesterol.—A 2.0 ml. aliquot of the alcohol extract in a 30 ml. Pyrex beaker is placed in a hood and when completely dry it is ready for color development. Two milliliters and 3.00 ml. of cholesteryl acetate working standard equivalent to 0.40 mg. and 0.60 mg. of cholesterol are treated similarly.

Free Cholesterol.—To a 3.0 ml. aliquot of the alcoholic extract in a 15 ml. Pyrex centrifuge tube* add 2 ml. of 95 per cent alcohol, 1 ml. of digitonin, 2 ml. of water, and one drop of 10 per cent acetic acid. A 3.0 ml. and a 5.00 ml. standard equivalent to 0.18 mg. and 0.30 mg. free cholesterol are set up simultaneously, omitting the 2 ml. of 95 per cent alcohol for the 5.0 ml. standard only. With a glass stirring rod for each tube, the reagents are mixed carefully and thoroughly, and with the glass rod in, the tube is covered to keep out dust and is allowed to stand overnight. With the glass rod, carefully mix into the supernatant any residue on the side of the tube. The glass rods are removed and placed on a holder, each rod reserved for its tube. The precipitate is sedimented by centrifuging at 2,750 r.p.m. for fifteen minutes, after which the supernatant is discarded. The glass rod is placed back into the tube, and 10 ml. of ether-acetone mixture 2:1 are added. The precipitate is carefully stirred and washed in the ether-acetone mixture. The glass rod is removed and placed on the holder as before. The precipitate is sedimented again at 2,750 r.p.m. for fifteen minutes. The ether-acetone washing of the unknown is carefully poured into a 50 ml. Pyrex beaker. Discard the washings from the standards. The glass rod is placed back into the tube and 5 ml. of ether are added. The precipitate is carefully stirred and washed in the ether. The glass rod is removed and placed on the holder as before. The precipitate is sedimented a third time by centrifuging at 2,750 r.p.m. for fifteen minutes. The ether washing is carefully added to the ether-acetone washing in the 50 ml. Pyrex beaker and both washings are saved for cholesterol ester determination. Again discard the washings from the standards. The glass rod is placed back into the tube, and the tube with the washed precipitate is set aside. When dry, 1.0 ml. of glacial acetic acid is added and the residue is dissolved by placing the tube in a bath of hot water for 5 minutes. The hot water is obtained by bringing it to a boil and then removing the heat just before the tube is placed into the water. The level of the water should be not more than 1 inch above the level of the glacial acetic acid in the centrifuge tube to prevent water from getting into the tube. When the solution is cool, it is ready for color development.

Ester Cholesterol.—The ether acetone and the ether washings are placed in a hood to evaporate. When dry, the esters are extracted twice by adding 15 ml. of petroleum ether and brought to a boil on a hot plate. This is decanted hot[4] into a 30 ml. Pyrex beaker and allowed to evaporate. When dry, it is ready for color development.

Total and Ester Cholesterol Color Development and Calculation.—To the beakers that contain standards and unknowns, 5.0 ml. of chloroform from a burette and 2.0 ml. of cold acetic anhydride sulfuric acid mixture are added and mixed. They are placed in the dark for fifteen minutes. The green color that develops is read in a Klett Summerson colorimeter with a green filter No. 54. The colorimeter is zeroed with a blank of 5.0 ml. of chloroform and 2.0 ml. of cold acetic anhydride sulfuric acid mixture.

$$\frac{\text{R unknown}}{\text{R standard}} \times 0.40 \times \frac{100}{0.20} = \text{mg. \% total cholesterol.}$$

$$\frac{\text{R unknown}}{\text{R standard}} \times 0.60 \times \frac{100}{0.30} = \text{mg. \% ester cholesterol.}$$

Free Cholesterol Color Development and Calculation.—Two milliliters of cold acetic anhydride sulfuric acid mixture are added to the tubes that contain the dissolved cholesterol digitonide and mixed with the stirring rod. After forty minutes in the dark, the stirring rod is removed and the green color that develops is read in a micro Klett tube on a Klett Summerson colorimeter with a green filter No. 54. The colorimeter is zeroed with a blank of 1.0 ml. of glacial acetic acid and 2.0 ml. of cold acetic anhydride sulfuric acid mixture.

*Wilson and Foldes[14] recommend a glass-stoppered centrifuge tube to eliminate the stirring rod.

$$\frac{\text{R unknown}}{\text{R standard}} \times 0.18 \times \frac{100}{0.30} = \text{mg. \% free cholesterol.}$$

$$\frac{\text{R unknown}}{\text{R standard}} \times 0.30 \times \frac{100}{0.30} = \text{mg. \% free cholesterol.}$$

EXPERIMENTAL

Method.—A better extraction of serum cholesterol is obtained from a fine dispersion. This takes place when alcohol is added to the serum[13] rather than the serum to alcohol.

Because the ratio of serum to solvent is greater than used in most procedures[1, 13, 15] a comparison between the amount of cholesterol extracted from 1 ml. of serum by 9.2 ml. of absolute alcohol (total volume 10 ml.) and the amount of cholesterol extracted from 1 ml. of serum by 24.2 ml. of absolute alcohol (total volume 25 ml.) has been made (Table I). The procedure as described under "*Extraction*" was followed using pooled serum. Table I shows the results obtained by heating in a water bath from thirty minutes at 84° C. and 56° C. The results indicate that at 84° C. or at 56° C. for thirty minutes, 9.2 ml. of absolute alcohol extract from 1 ml. of serum the same amount of cholesterol that is extracted by 24.2 ml. of serum.

TABLE I. COMPARISON OF CHOLESTEROL EXTRACTION FROM 1 ML. OF SERUM USING 9.2 ML. AND 24.2 ML. OF ABSOLUTE ALCOHOL

ALCOHOL VOL.	MG. PER CENT CHOLESTEROL (AVERAGE OF DUPLICATE)			
	A	B	C	D
	Temp. 84° C.	*Time 30 min.*		
9.2 ml.	215	244	186	201
24.2 ml.	215	242	183	201
	Temp. 56° C.	*Time 30 min.*		
	E	F	G	
9.2 ml.	192	260	217	
24.2 ml.	192	264	217	

Two normal sera (195 mg. per cent and 225 mg. per cent) and one very high cholesterol serum (593 mg. per cent) were used to determine the length of time necessary to extract all of the cholesterol from 1 ml. of serum at 56° C., using only 9.2 ml. of absolute alcohol. Table II shows that ten minutes extracts the same amount of cholesterol as thirty and sixty minutes.

TABLE II. EFFECT OF TIME OF EXTRACTION

1 ML. OF SERUM AND 9.2 ML. ABSOLUTE ALCOHOL AT 56° C.						
	MG. PER CENT CHOLESTEROL					
TIME	A		B		C	
10 min.	198	196	225	224	594	600
30 min.	186	192	222	226	586	593
60 min.	194	196	229	224	586	600

All drying is done by air draft at room temperature. Large discrepancies were found between duplicates when extracts were dried on the steam bath. Bergstrom and Wintersteiner[16] have shown that cholesterol when aerated at

85° C. in colloidal aqueous solution is for the major part auto-oxidized into a mixture of compounds. The method for determining free cholesterol is essentially that of Schoenheimer and Sperry[1] with variations in the digitonide reagent[12, 13] and the per cent water[17] for cholesterol digitonide precipitation. The use of larger amounts of ether acetone mixture and ether for washing eliminated one ether washing. The supernatant of the digitonide precipitate from serum does not contain a detectable amount of cholesterol.

This supernatant from several sera having cholesterol levels ranging from 170 mg. per cent to 400 mg. per cent was saved for analysis. The residue remaining after evaporation was extracted with petroleum ether. This extract was dried and treated with acetic anhydride sulfuric acid reagent in the usual manner. The solutions appeared water white and gave a zero reading against the blank on the colorimeter.

Cholesterol as cholesteryl acetate gives a color, measured at 540 μ, that is approximately 7 per cent more intense than the same amount of cholesterol. This was obtained as the average of thirteen comparisons, in duplicate, on as many different days. The average deviation was 1.23 per cent. This discrepancy is much less than that reported by other workers when wave lengths of 660, 640, and 420 μ were used. Differences of 12 to 30 per cent[18-21] have been reported for various cholesteryl esters. The increase in color produced by the acetate very closely equals the increased color from the esters present in the serum when measured at 540 μ. For this reason values obtained by using pure cholesteryl acetate as a standard are equal to the values obtained by the Schoenheimer and Sperry method.

Determination of Accuracy of Method by Recovery Experiments.—Cholesterol and cholesterol-cholesteryl acetate in varying amounts and ratios were added to 1.0 ml. of fresh pooled serum after it had been mixed with 8.2 ml. of alcohol. The average recovery (Table III) of cholesterol added (0.40 mg. to 3.00 mg.) as total cholesterol for fourteen determinations was 97 ± 3.3 per cent. The average recovery of cholesterol added (0.12 mg. to 2.00 mg.) as free cholesterol for nine determinations was 101 ± 3.7 per cent. Cholesteryl acetate could be recovered quantitatively only as total cholesterol. However, in serum alone, the esters are quantitatively determined because when they are added to the free cholesterol the sum equals the total cholesterol.

The accuracy of this method is further emphasized by the consistency of duplicates (94 of 100 determinations of total cholesterol in duplicate differed by 3 per cent or less) and by the fact that the value obtained for total cholesterol is equal to the sum of the values of ester and free cholesterol. In seventy-eight of eighty-five serums, in duplicate, the total cholesterol exceeded or was less than the sum of the esters and the free cholesterol by not more than 3 per cent. The mean deviation and standard deviation were ± 0.15 per cent, and ± 1.91 per cent, respectively.

Comparison Between Authors' and Schoenheimer and Sperry's[1] Methods.—The use of cholesteryl acetate as a standard and the method of extraction were checked by comparing total cholesterol determined by our method with total cholesterol determined by Schoenheimer and Sperry's method[1] (Table IV).

TABLE III. RECOVERY OF CHOLESTEROL AND CHOLESTEROL ACETATE ADDED TO 1.0 ML. OF POOLED SERUM

SAMPLE	ORIGINAL (MG.)	ADDED TOTAL CHOLESTEROL (MG.)	RECOVERED IN SERUM (MG.)	RECOVERY (PER CENT)
1	2.64	0.50	0.52	104
2	2.64	1.00	1.01	101
3	2.64	1.00	1.01	101
4	2.64	2.00	1.86	93
5	2.04	0.40	0.40	100
6	2.04	0.80	0.76	95
7	2.04	1.20	1.10	92
8	2.28	1.00	0.91	91
9	2.28	2.00	2.00	100
10	2.28	3.00	2.91	97
11	2.64	2.00	1.88	94
12	2.04	1.00	0.94	94
13	2.04	2.00	1.96	98
14	2.04	3.00	2.88	96
Average				97 ± 3.3
		Free Cholesterol in Serum		
5	0.56	0.12	0.11	92
6	0.56	0.24	0.25	104
7	0.56	0.36	0.36	100
8	0.55	0.30	0.31	104
9	0.55	0.60	0.61	102
10	0.55	0.90	0.94	105
12	0.57	0.50	0.48	96
13	0.57	1.00	1.02	102
14	0.57	1.50	1.60	107
Average				101 ± 3.7

Samples 1-3 solution added was cholesterol.
Samples 4-10 solution added was 30 per cent cholesterol and 70 per cent cholesteryl acetate.
Samples 11-14 solution added was equal parts cholesterol and cholesteryl acetate.

The group average of 226 mg. per cent by the authors' method checks well with the 225 mg. per cent by the Schoenheimer and Sperry method.

Effect of Time and Temperature on Color Development.—Two milliliters of cholesteryl acetate standard equivalent to 0.40 mg. of cholesterol were evaporated to dryness in a 30 ml. Pyrex beaker and the color was developed as directed in the procedure. The solutions remained in the light after the first

TABLE IV. COMPARISON OF TOTAL CHOLESTEROL BETWEEN AUTHORS' AND SCHOENHEIMER AND SPERRY'S[1] METHODS

SAMPLE NO.	AUTHORS' TOTAL AVERAGE (MG. PER CENT)	AUTHORS' NUMBER OF DETERMINATIONS	SCHOENHEIMER AND SPERRY TOTAL AVERAGE (MG. PER CENT)	SCHOENHEIMER AND SPERRY NUMBER OF DETERMINATIONS
1	214	4	214	6
2	166	4	158	2
3	265	6	265	5
4	183	2	184	4
5	286	4	291	8
6	230	6	226	6
7	227	4	228	3
8	215	4	213	5
9	248	4	248	6
Average	226		225	

TABLE V. EFFECT OF TIME AND TEMPERATURE ON COLOR DEVELOPMENT IN CHLOROFORM. TOTAL AND ESTER STANDARD. 2.0 ML. OF CHOLESTERYL ACETATE STANDARD EQUIVALENT TO 0.40 MG. OF CHOLESTEROL

TIME (MINUTES)	TEMPERATURE				
	17° C.	21° C.	26° C.	29° C.	37° C.
	Colorimeter Readings				
10	96	91.5	87	85	82
20	95	90.5	84	81	75.5
30	89.5	88	82.5	80	74.5
40	88.5	87.5	81.5	79.5	74

reading. The colorimeter reading's average of duplicates, decreased with time and temperature (Table V). The difference between 21° C. and 29° C. at fifteen minutes and at forty minutes is one colorimeter reading per degree of temperature.

The effect of time and temperature on the color developed with the standard dissolved in acetic acid is shown in Table VI. At temperatures just above 24° C., the standard reaches a maximum in forty minutes and remains so for at least twenty minutes.

TABLE VI. EFFECT OF TIME AND TEMPERATURE ON COLOR DEVELOPMENT IN ACETIC ACID. FREE CHOLESTEROL STANDARD. 3.0 ML. OF CHOLESTEROL WORKING STANDARD EQUIVALENT TO 0.18 MG. OF CHOLESTEROL

TIME (MINUTES)	TEMPERATURE				
	17° C.	21° C.	24° C.	27° C.	37° C.
	Colorimeter Readings				
40	44	70	72	75	74
50	62	72	75	76	75
60	72	75	75	75	75

Table VII shows the cholesterol values of a series of serum extracts determined at ten-minute intervals. The series was prepared from a pooled alcoholic extract of three different serums. Ten 2.0 ml. aliquots of the unknown alcoholic extract and ten 2.0 ml. aliquots of cholesteryl acetate, in 30 ml. Pyrex beakers, were dried and the color was developed at room temperature (24° C.) as directed in the procedure. At ten-minute intervals two unknowns and two standards were removed from the dark and compared in the colorimeter. The variation from ten to fifty minutes was 224 to 232 mg. per cent, and the maximum deviation between any duplicates was 224 to 232 mg. per cent. The average of these ten values is 227.2 ± 2.4 mg. per cent, and the average of the values determined individually of the three serums from which

TABLE VII. EFFECT OF TIME ON THE TOTAL CHOLESTEROL OF A POOLED ALCOHOLIC EXTRACT OF THREE SERUMS

DUPLICATE	TIME IN MINUTES (TEMPERATURE 24° C.)				
	10 (MG. PER CENT)	20 (MG. PER CENT)	30 (MG. PER CENT)	40 (MG. PER CENT)	50 (MG. PER CENT)
1	227	232	228	229	224
2	227	224	225	232	224

TABLE VIII. PARTITION OF SERUM CHOLESTEROL IN FASTING NORMAL ADULTS

	CHOLESTEROL						
SAMPLE	TOTAL (MG. PER CENT)	ESTER (MG. PER CENT)	FREE (MG. PER CENT)	ESTER ± FREE (MG. PER CENT)	EST./TOT. (PER CENT)	FR./TOT. (PER CENT)	ESTER ± FREE (PER CENT)
1	270	189	72	261	70	27	97
2	248	182	63	245	73	25	98
3	248	180	64	244	73	26	99
4	240	175	68	246	73	28	101
5	235	161	76	237	69	32	101
6	235	164	67	231	70	29	99
7	230	178	56	234	77	24	101
8	218	157	58	215	72	27	99
9	214	152	66	218	71	31	102
10	213	150	65	215	70	30	100
11	212	159	57	216	75	27	102
12	210	156	57	213	74	27	101
13	208	154	54	208	74	26	100
14	206	146	60	206	70	30	100
15	201	152	48	200	76	24	100
16	198	139	60	199	70	30	100
17	197	134	59	193	68	30	98
18	194	140	48	188	72	25	97
19	191	143	49	192	75	26	101
20	180	132	49	181	73	27	100
21	177	127	51	178	72	29	101
22	176	130	48	178	74	27	101
23	175	129	50	179	74	29	103
24	158	114	46	160	72	29	101
25	153	105	44	149	69	29	98
Average	207.5	149.9	57.6	207.5	72.2	27.8	100.0

the pooled alcoholic extract was obtained is 228.3 mg. per cent. Since the readings of both standard and unknown decrease similarly with time, a duplicate standard can be effectively spaced among a long series of unknowns for more accurate results. In this manner, it was possible to eliminate both the regulation of temperature and the timing of individual samples.

Determination of Normal Range of Serum Total Cholesterol, Cholesterol Esters, and Free Cholesterol.—Determination of serum total cholesterol, cholesterol esters, and free cholesterol on twenty-five fasting normal adults are shown in Table VIII. These values are the average of duplicate serum extracts. The average total (207.5 mg. per cent) is slightly less than the 209.8 mg. per cent

TABLE IX. PARTITION OF SERUM CHOLESTEROL IN FASTING ADULTS WITH ABNORMAL CONDITIONS EFFECTING SERUM CHOLESTEROL

	CHOLESTEROL						
SAMPLE	TOTAL (MG. PER CENT)	ESTER (MG. PER CENT)	FREE (MG. PER CENT)	ESTER ± FREE (MG. PER CENT)	EST./TOT. (PER CENT)	FR./TOT. (PER CENT)	ESTER ± FREE (PER CENT)
26	196	100	94	194	51	48	99
27	125	61	63	124	49	50	99
28	185	57	127	184	31	69	100
29*	408	98	238	336	29*	70	99
30	507	92	407	499	18	80	98

*We noted that this patient had a total bilirubin of 11.2 mg. per cent by the method of Evelyn and Malloy. A saponified total was 339 mg. per cent and was used to determine per cent esters and per cent free.

found by Sperry.[22] The average free per cent (27.8 per cent) is almost the same as the 26.9 ± 1.4 per cent found by Sperry.[22] The average ester per cent is the same as the 72 per cent found by molecular distillation of serum cholesterol esters at 128° C. as reported by Koehler.[23]

Cholesterol Partition of Abnormal Sera.—The serum cholesterol with abnormal partition or of unusually high concentration is of particular interest in checking the accuracy of a method. Table IX shows several such sera with unusually high free cholesterol content. In these sera, as well as those of normal partition and concentration, the sum of the ester and free values equals the values obtained for the total. The one exception (No. 29) had an unusually high icteric index; however, when the total cholesterol was repeated, including a saponification step, the results of this saponified specimen equaled the sum of the ester and the free cholesterol.

SUMMARY

A procedure for the determination of total, ester, and free cholesterol is presented that is fairly rapid and as accurate as the more tedious methods currently available.

The salient points of the procedure are:

Use of the 540 absorption range on a filter type photocolorimeter, thereby avoiding temperature control and individual specimen timing.

Use of cholesteryl acetate as the reference standard, thereby eliminating saponification.

The sum of the ester and free cholesterol values is within 3 per cent of the value obtained for the total cholesterol.

The determination of total cholesterol is simplified.

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AN APPARATUS FOR INCREASING THE SPEED OF DIALYSIS OF SMALL QUANTITIES OF FLUIDS

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DURING the course of a fairly extensive screening program in which the efficacy of many substances in precipitating certain proteins from mixtures was tested, it became necessary to dialyze small quantities of many solutions. In order to speed up the attainment of equilibrium between the solutions being dialyzed and the bath in which they were dialyzing, an apparatus was employed which brings about continued turnover of the solutions on both sides of the interface.

MATERIALS

The device, shown in Fig. 1, is constructed entirely of plexiglas to avoid metal contamination. The heads of necessary bolts are coated with plexiglas cement. Two identical wheels, six inches in diameter and 0.75 inch thick, are rigidly fastened, twelve inches apart, to a shaft 0.75 inch in diameter. The pegs onto which the dialysis sacs are tied are 0.5 inch deep and are evenly spaced around the circumference. The pegs are tapered as shown so that the sacs cannot slip off during rotation. Our machine carries sixteen such pegs on each wheel. At this spacing, it is possible to use every peg when using dialysis tubing 24/32 inches or smaller; if larger tubing is used, only alternate pegs can be employed. To avoid confusion, the pegs on only one wheel are numbered (numbers are easily scratched into the plexiglas). The shaft also carries a grooved pulley, which permits rotation by means of a belt extending to a motor suspended above the device and resting on the sink edges.

Although the exact dimensions of the machine will vary, depending on the size of the sink to be used and by the quantities of fluid to be dialyzed, the dimensions quoted are for a machine used in a rectangular sink 17½ inches wide, 21½ inches in length, and 12 inches deep. In use, the drain of the sink is stopped with a one-hole rubber stopper bearing a large, thick-walled glass tube approximately seven inches high. This tube permits constant level overflow if a trickle of fresh water is maintained. A bath is thus formed of about 45 liters.

The pulley belt is fabric composition. Dialysis tubing used is Visking cellulose tubing, each tube containing an acid-washed glass marble. Both ends of the tube are tied up with heavy linen thread, and the filled tubes are fastened to the machine with linen thread in the case of the smaller sized tubes (0.5 inch or less) and with twine in the case of larger tubes. Thus the only contamination in the bath is the small amount of "sizing" material on some samples of twine. Because of the sizing, we ordinarily fill the bath, immerse the loaded machine for several minutes, then empty and refill the bath.

EXPERIMENTAL AND DISCUSSION

The general principle of the apparatus is the slow rotation about a horizontal axis, of tubes fixed at an angle to the horizontal. Each tube contains a loosely fitting glass marble which, rolling up and down the tube as it rotates

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at its angle of 30 to 40 degrees from the axis, continually renews the solution layer immediately inside the tube. At the same time, the rotation in the bath presents a continual fresh surface on the outside of the tube.

Factors of importance are: (a) angle of tilt, (b) viscosity of dialyzing fluid, (c) speed of rotation, and (d) the relative size of the tube and the glass marble. A too-large marble will not traverse the length of the tube; a too-small one will not effectively renew the inner surface layer. With a lesser angle of tilt, or a greater viscosity of solution, the speed of rotation must be decreased to allow time for complete traversal by the marble.

Our motor is so geared as to rotate the tubes at approximately four revolutions per minute. Two sizes of dialysis tubing most commonly used are 24/32 and 36/32 inch in diameter. These sizes were selected because the proper sized glass marbles, approximately $\frac{5}{8}$ inch and 1 inch in diameter, are commonly available in five-and-ten cent stores.

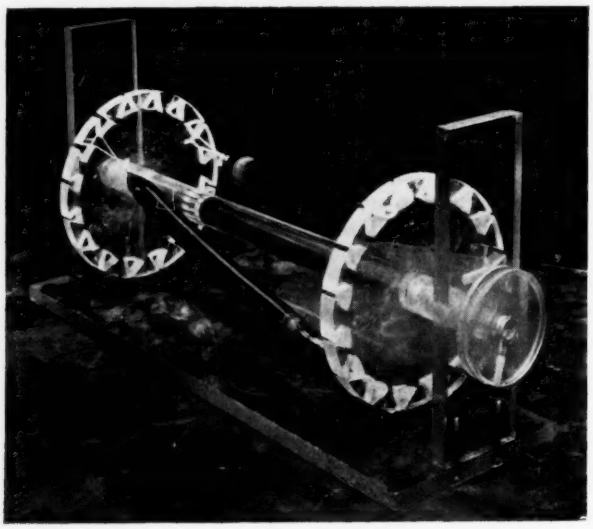

Fig. 1.

When 24/32 inch tubing is used, the machine can accept tubes containing a maximum of approximately 75 ml. each. Tubes 36/32 inch in diameter contain as much as approximately 160 ml. One limitation that should be noted is that if a wide variety of substances is being simultaneously dialyzed, care must be taken that sharp incompatibilities are not permitted. It is obvious, of course, that equilibrium is attained not only between the dialyzable contents of each tube and the bath, but between *all* dialyzable materials, in and out of the sacs. Ordinarily this is not of great concern, chiefly because of the very great dilution brought about by the bath water.

In order to test the efficiency of the machine, the following experiment was performed. Seven dialysis sacs, 20/32 inches in diameter, approximately eight inches in length, and each containing a glass marble about 0.5 inch in diameter

were filled with $(NH_4)_2SO_4$ solution, approximately 25 per cent saturated. These were placed on the machine and rotated in the sink bath described; no running water was permitted. The tubes were removed at various times, emptied into beakers, and the contents were analyzed for nitrogen by direct nesslerization. On another day, but using the same solution of $(NH_4)_2SO_4$, the same sink, similarly filled, and the same size dialysis sac and marbles, another seven tubes were dialyzed. These, however, floated free in the bath, supported by a holiow weighing bottle stopper. According to common practice, they were inverted by hand every half hour or so during the working day. Individual tubes were removed at various times, and aliquots of the contents nesslerized. The results are shown in Table I. It is seen that attainment of equilibrium was considerably speeded by the use of the machine.

TABLE I. RELATIVE EFFICIENCY OF STATIC AND ROTATING DIALYSIS

TIME IN BATH (HR.)	MG. N PER ML. SOLUTION IN DIALYSIS SAC	
	FREE-FLOATING	ON MACHINE
0	22.8	22.3
0.5	15.6	12.5
1	10.5	8.2
2	3.0	2.1
4	1.0	0.4
6	0.5	0.1
24	0.1	0.1

SUMMARY

An apparatus is described which assists in the rapid attainment of equilibrium in the dialysis of small quantities of solutions. In the apparatus described, a maximum of sixteen solutions, each about 75 ml. or less, can be simultaneously dialyzed.

THE INFLUENCE OF DIFFERENT TYPES OF CORN MEAL AGAR UPON CHLAMYDOSPORE PRODUCTION BY CANDIDA ALBICANS

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CORN meal agar came into general use for the identification of *Candida albicans* following Benham's[1] account, in 1931, of its role in the stimulation of characteristic mycelial growth and chlamydospore production by this fungus. Linossier and Roux,[2] in 1889, had observed structures which they called chlamydospores in *C. albicans* grown at 30 to 35° C. in a liquid medium containing saccharose, ammonium tartrate, potassium phosphate, magnesium sulfate, and calcium chloride. Langeron and Talice[3] later (1932) employed potato water, and Shrewsbury,[4] in 1934, obtained such spores in milk-water and in boiled blood broth. Anderson,[5] in 1946, used corn meal extract to which vital dyes were added.

Benham[1] described a direct slide technique for the determination of mycelium and chlamydospores, as well as a Petri dish method wherein cultures are made by cutting slits across a deep layer of the medium with an inoculating needle and studied by placing a cover slip directly on the slit. She gave her procedure for the preparation of the corn meal agar, but specified no particular type of corn meal. Some later authors have recommended yellow corn meal.[6-9] Conant and associates[10] state that *C. albicans* will not produce chlamydospores regularly on Bacto-corn meal agar (which contains 0.2 per cent dextrose), and Skinner[11] warns that if one uses a dehydrated medium he should make certain that it contains no glucose. There does not, however, appear to have been any large-scale comparative study of different types of corn meal with respect to color, additives, or method of preparation.

In addition to the problem of dehydrated versus homemade media, the question arose whether vitamin-enriched yellow corn meal, which has been in routine use in this laboratory because the unenriched yellow kind is unavailable locally, might be inferior to the latter, since mycelial and chlamydospore production have been presumed to be encouraged by nutritionally impoverished media. A study was therefore undertaken of the comparative efficacy of four different kinds of corn meal agar for the morphological identification of routinely isolated strains of *C. albicans*.

MATERIALS AND METHODS

Three of the test media were homemade, being prepared in identical manner except for the type of corn meal employed. These types were: (1) yellow enriched (Quaker Yellow Enriched Degerminated Corn Meal), manufactured by the Quaker Oats Co. and obtained com-

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mercially. This product contains vitamins B_1, B_2, niacin, and iron; (2) yellow unenriched, kindly supplied by Dr. Elizabeth L. Hazen of the New York State Department of Health; (3) white unenriched, prepared by a local milling company and obtained commercially.

In the preparation of these media, 40 Gm. of corn meal was simmered in one liter of distilled water at 65° C. for one hour, and filtered successively through eight layers of gauze and through coarse filter paper (E & D folded filter paper No. 192). The volume of filtrate was measured and restored to one liter. Twenty grams of Bacto-agar was then added and the suspension autoclaved for five minutes to dissolve the agar. The pH was adjusted as required, the media dispensed in flasks, and autoclaved fifteen minutes at 120° C.

The fourth medium was "Bacto-corn Meal Agar Special," supplied to this laboratory for trial by Mr. H. W. Schoenlein of Difco Laboratories. This dehydrated medium was stated to contain yellow unenriched corn meal without added dextrose. It was rehydrated by suspension of 17 Gm. of the powder in one liter of cold distilled water, heated to boiling, the pH adjusted, dispensed in flasks, and autoclaved fifteen minutes at 120° C.

Prior to use, all media were melted in a water bath, cooled, and poured into Petri dishes in quantities of approximately 25 ml.

The final pH of the first two media, without adjustment, was 6.6. That of the latter two, initially 5.6, was adjusted to 6.6.

Each of 145 unselected strains of *C. albicans*, all routine isolates from clinical materials submitted to this laboratory, was inoculated into all four media. The method of isolation and identification of these strains was as follows: a suspected colony on the primary isolation medium (Sabouraud dextrose agar with or without added penicillin and streptomycin, or blood agar with the two antibiotics) was transferred to Sabouraud dextrose broth and incubated at 37° C. for twenty-four to forty-eight hours; a blood agar plate was streaked from the broth, and after forty-eight hours or longer, single, well-isolated colonies were transferred to Sabouraud dextrose agar slants; inocula from these were then cut into routine yellow enriched corn meal agar plates. The latter were incubated at 25° C. and read at forty-eight or seventy-two hours for presence of pseudohyphae and chlamydospores. Isolates otherwise morphologically typical of *C. albicans* but not producing chlamydospores on the corn meal agar after seventy-two hours were transferred therefrom to a second such plate and the readings repeated. Those identified, on the basis of chlamydospore production, as *C. albicans* were maintained on Sabouraud dextrose agar slants until used in the tests.

For the comparison of media a twenty-four hour subculture of each test strain was inoculated into one plate of each of the four kinds of corn meal agar. Small but visible quantities of inoculum were taken on the end of a needle and duplicate cuts, about one inch apart, were made in parallel streaks from edge to edge across the center of the plate, going completely through the agar. A sterile 22 by 40 mm. cover glass was then placed across each pair of cuts, about one-half inch from the points of inoculation. The plates were incubated at 25° C. in the dark and read at twenty-four and forty-eight hours. If no chlamydospores appeared on any of the plates at this time, they were read again at seventy-two hours. Quantitative estimates of the amount of mycelium produced along the streaks and of the number of chlamydospores in relation to the *amount of mycelium* were recorded on a 1- to 4-plus scale. Readings were made with the low power of the compound microscope (×100) through the inverted bottom of the unopened plate, as is done routinely in this laboratory. The area under the cover glass was then examined, with the plate right side up and lid removed, and note was made of the difference, if any, in the quantitative reading obtained by the two methods.

As a final check on the identity and purity of the cultures employed in this experiment, all strains were saved and later put through an identification procedure modified from that of Jones and Martin.[12] Each strain was transferred from Sabouraud dextrose agar to Sabouraud dextrose broth and incubated at 37° C. for forty-eight hours. The presence or absence and type of surface growth were recorded, the tube shaken, and a loopful of the yeast suspension streaked on a blood agar plate, which was incubated at 37° C. for several days. The type of growth produced was recorded and a single colony transplanted again to a Sabouraud slant. After forty-eight hours at 37° C., growth from the slant was streaked

on a beef extract agar slant (sugar-free), from which two additional serial subcultures were made on the same medium. The last beef extract tube was then used to inoculate four beef extract broth tubes containing, respectively, one per cent of dextrose, maltose, sucrose and lactose. The sugar tubes were sealed with sterile petrolatum and incubated at 37° C. for ten days.

RESULTS

Results of the corn meal agar test readings for chlamydospores and hyphae are summarized in Table I. All but two of the 145 strains produced chlamydospores on at least one type of corn meal, but individually the best of the four types evoked their production in only 82 per cent of the strains (119 of 145). Included in the table are four strains which, having failed to produce chlamydospores on any medium after forty-eight hours, were retested. At the conclusion of the repeat test two of these strains gave readings of 1-plus on one and two of the media, respectively, while the others remained negative on all of the media. In quantitative terms, about the same proportion of positives were read as 4-plus on both the yellow enriched (55 of 112) and yellow unenriched (63 of 119) corn meal agars. Much lower percentages were recorded for the Difco Special (28 of 85) and white (24 of 84) corn meal media.

TABLE I. PRODUCTION BY CANDIDA ALBICANS OF HYPHAE OR OF HYPHAE AND CHLAMYDOSPORES ON SPECIFIED TYPES OF CORN MEAL AGAR

	HYPHAE	HYPHAE AND CHLAMYDOSPORES
Total strains tested	145	145
Number of strains producing specified structures on:		
at least one of the 4 types of medium	145	143
Yellow enriched corn meal agar	142	112
Yellow unenriched corn meal agar	142	119
Difco Special corn meal agar	143	85
White unenriched corn meal agar	138	84

Each of the strains employed produced hyphae on at least one type of corn meal in its respective lot, and few failed to exhibit hyphae on every medium. However, the proportion of 3- and 4-plus determinations to the total was much higher on yellow enriched and yellow unenriched than on the other two media.

All but two of the 145 strains included in the table exhibited the typical *C. albicans* pattern of sugar fermentations, with acid and gas in both dextrose and maltose and acid alone, varying from very slightly to frankly acid, in sucrose. Lactose was never fermented. The two aberrant isolates failed to produce acid in sucrose, but were otherwise consistent. All strains formed typical colonies on blood agar plates and none showed surface growth on Sabouraud broth at forty-eight hours, although several produced a slight film on the side of the tube after prolonged incubation.

DISCUSSION

None of the corn meal agars tested appears to be the ideal medium for eliciting chlamydospore production by *C. albicans*. Yellow enriched and yellow unenriched corn meal were fairly reliable and about equal in this respect while, in these experiments, white corn meal and Difco Special were found to be

inferior. With each medium, there was considerable variation among the results obtained on separate lots prepared from the same box of corn meal. Strain differences constitute one of the most important factors influencing the results, and any new medium designed to improve upon the above will have to minimize these differences. It should be remembered that, in addition to chlamydospores, presence or absence of hyphae on corn meal plates is of prime diagnostic significance, since the medium serves not only to differentiate *C. albicans* from the other species of *Candida*, but also to aid in the identification of such additional yeastlike organisms as *Cryptococcus*.

Possibly the percentage of chlamydospore positives would have been higher had the inocula for the corn meal cuts been taken from sugar-free media, as advocated by Jones and Martin.[12] However, their method is a lengthy one, and the present study was an attempt to evaluate corn meal media by the rapid identification procedure in routine use. Nickerson,[13] in working with chemically-defined media, concluded that production of chlamydospores on his test medium depended upon absence of glucose from the substrate.

For the routine laboratory identification of *C. albicans* by the corn meal agar plate method it is important that a known chlamydospore-producing strain be cut into each plate as a control, that the plates be incubated at room temperature (about 25° C.) rather than at 37° C., and that readings not be postponed much beyond forty-eight hours, although frequently chlamydospores will not appear until after three days. When incubation is prolonged there is a tendency by the blastospores to proliferate, thus obscuring the diagnostic chlamydospores. It has been the practice in this laboratory to examine corn meal agar plates, which, if properly prepared, are quite clear and transparent, by inverting the unopened dish upon the compound microscope stage and using the low-power objective, as suggested by Conant and associates.[10] During the present experiments the use of a cover glass placed atop the corn meal cuts, as advocated by Kligman,[14] displayed no advantage over the inverted dish method so far as production of chlamydospores was concerned. The cover glass procedure undoubtedly permits better photography, if such is contemplated, but also exposes the medium to contamination, thus hindering any necessary subsequent examination.

The depth of agar in the corn meal plate is also of some concern. It has been found that the optimum volume in an ordinary Petri dish (100 mm.) is 25 to 30 ml. Thinner plates do not permit as rapid formation of chlamydospores, while additional agar tends to obscure the structures.

SUMMARY AND CONCLUSIONS

Four different types of corn meal agar were tested with 145 strains of *Candida albicans* for their ability to permit rapid identification of this organism. Under the conditions of this experiment, yellow enriched and yellow unenriched corn meal agar, both homemade, were found to be about equally effective and superior to homemade white corn meal agar and to Difco Special

corn meal agar for the production of chlamydospores by this fungus. All four media regularly permitted mycelial formation, although the two former appeared to be superior quantitatively in this respect.

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